UNIVERSIDAD COMPLUTENSE DE MADRID

FACULTAD DE CIENCIAS QUÍMICAS
Departamento de Bioquímica y Biología Molecular I

RIBOTOXINAS FÚNGICAS: DEL ANÁLISIS MOLECULAR DE SU MECANISMO CITOTÓXICO A SUS POSIBLES APLICACIONES CLÍNICAS.

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Bajo la dirección de los doctores

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Ribotoxinas fúngicas: del análisis molecular de su mecanismo citotóxico a sus posibles aplicaciones clínicas

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TESIS DOCTORAL

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ABREVIATURAS

ABPA	Aspergilosis broncopulmonar alérgica
ATR	Reflexión total atenuada
CD	Dicroísmo circular
cDNA	DNA codificante, sintetizado utilizando RNA como molde
DMPG	Dimiristoilfosfatidilglicerol
DT	Toxina de la difteria
ELISA	*Enzyme-linked immunosorbent assay*
EF	Factor de elongación
FTIR	Espectroscopía de infrarrojo asistida por transformada de Fourier
G	Guanina
GRAS	Generalmente considerada segura (*generally regarded as safe*)
HPLC	Cromatografía líquida de alta eficiencia (*high performance liquid cromatography*)
HtA	Hirsutelina A
Ig	Inmunoglobulina
IMTX	Inmunotoxina
mAb	Anticuerpo monoclonal
PAGE	Electroforesis en geles de poliacrilamida
PBS	Tampón fosfato salino
PCR	Reacción en cadena de la polimerasa
PDB	*Protein data bank*
PE	Exotoxina de *Pseudomonas*
pI	Punto isoeléctrico

RIP	Proteína inactivadora de ribosomas
RMN	Resonancia magnética nuclear
rRNA	Ácido ribonucleico ribosomal
RNasa	Ribonucleasa
SDS	Dodecil sulfato sódico
SIT	Inmunoterapia alérgeno-específica
SRL	Lazo sarcina-ricina
TA	Toxina-antitoxina
TFR	Receptor de transferrina
tRNA	RNA de transferencia
UV	Ultravioleta

Introducción

Ribotoxinas: análisis molecular de una familia de ribonucleasas fúngicas

Resumen

La ribonucleasa T1 es la proteína más representativa y mejor conocida de toda una gran familia de ribonucleasas secretadas por hongos, principalmente por especies de los géneros *Aspergillus* y *Penicillium*. Dentro de esta familia destacan las ribotoxinas por su carácter citotóxico, que se basa en dos actividades independientes. Por una parte, son capaces de atravesar membranas lipídicas y entrar en las células y por otra, poseen una actividad ribonucleolítica con la que rompen un único enlace fosfodiéster localizado en una secuencia universalmente conservada dentro del mayor fragmento del RNA ribosomal, conocida como lazo sarcina/ricina. De esta forma inhiben la biosíntesis de proteínas, provocando la muerte celular por apoptosis. No se ha encontrado ningún receptor proteico para las ribotoxinas; en cambio, estas proteínas atacan principalmente a células con una permeabilidad de membrana alterada, como aquellas infectadas por virus o transformadas. Gracias a una amplia variedad de aproximaciones metodológicas y a la construcción y purificación de diferentes versiones mutantes de estas ribotoxinas se ha conseguido dilucidar en gran parte su mecanismo citotóxico a nivel molecular. Haciendo uso de su toxicidad, las ribotoxinas se han utilizado en la construcción de inmunotoxinas. Por otra parte, se ha demostrado que Asp f 1, una ribotoxina producida por *Aspergillus fumigatus*, es uno de los principales alérgenos implicados en patologías relacionadas con la aspergilosis alérgica. La ingeniería de proteínas y la síntesis de péptidos han permitido comprender las bases de estos mecanismos patogénicos así como producir proteínas hipoalergénicas con potenciales aplicaciones diagnósticas e inmunoterapéuticas.

Introducción

Las ribotoxinas son una familia de ribonucleasas (RNasas) fúngicas, extracelulares y citotóxicas, que ejercen su actividad ribonucleolítica sobre la mayor molécula de RNA del ribosoma, provocando la inhibición de la biosíntesis de proteínas y la muerte celular por apoptosis (Gasset *et al.*, 1994; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). Varios estudios han propuesto que existe una relación entre su localización en la superficie de los conidióforos fúngicos y la maduración de los conidios, lo que sugiere que su producción podría estar relacionada con una función defensiva para los hongos a la hora de reproducirse (Brandhorst y Kenealy, 1992; Yang y Kenealy, 1992a,b). Las ribotoxinas fueron descubiertas durante un programa de búsqueda de antibióticos y agentes antitumorales iniciado en 1956 en el Departamento de Salud de Michigan. Se observó que el filtrado del medio de cultivo de un hongo aislado del suelo de una granja de Michigan contenía una sustancia inhibitoria tanto del sarcoma 180 como del carcinoma 755 inducidos en ratón. (Olson *et al.*, 1965b). El hongo fue identificado como *Aspergillus giganteus* MDH18894 (Figura 1a), y se demostró que la molécula responsable del efecto era una proteína, que se denominó α-sarcina (Figura 1b) (Olson y Goerner, 1965). Más tarde se observó que dos proteínas antitumorales más, restrictocina y mitogilina, ambas producidas por *A. restrictus*, presentaban actividades similares a la α-sarcina, y fueron por tanto incluidas en el mismo grupo de moléculas antitumorales. Asp f 1, otra ribotoxina, producida por *Aspergillus fumigatus*, fue identificada mucho después como un alérgeno principal en las enfermedades relacionadas con *Aspergillus* (Arruda *et al.*, 1992). Desafortunadamente, estudios posteriores desvelaron la citotoxicidad inespecífica de estas proteínas, lo que limitó sus potenciales usos clínicos (Roga *et al.*, 1971). Así, el estudio de estas toxinas se abandonó hasta la mitad de la década de los 70, cuando se descubrió que inhibían la biosíntesis de proteínas a concentraciones tan bajas como 0.1 nM, debido a la ruptura específica de un único enlace fosfodiéster del RNA ribosomal (rRNA) mayor (Schindler y Davies, 1977; Endo y Wool, 1982). De hecho, la actividad específica de las ribotoxinas es tan efectiva que basta una sola molécula de α-sarcina para matar a una célula (Lamy *et al.*, 1992). El enlace que rompen las ribotoxinas es especialmente interesante por su localización en el lazo sarcina/ricina (SRL), una región evolutivamente conservada con papeles importantes en la funcionalidad del ribosoma, pues parece estar relacionada con la unión de aminoacil-tRNA dependiente del factor de elongación 1 (EF-1) y la hidrólisis de GTP y posterior translocación dependientes de EF-2 (Wool *et al.*, 1992).

Además de poseer esta actividad ribonucleolítica, las ribotoxinas son capaces de atravesar membranas lipídicas en ausencia de receptores proteicos (Oñaderra *et al.*, 1993; Gasset *et al.*, 1994; Martínez-Ruiz *et al.*, 2001) y por este motivo, aunque potencialmente cualquier ribosoma podría ser inactivado por estas proteínas dado que el SRL está universalmente conservado, las ribotoxinas son especialmente activas sobre células transformadas o infectadas por virus (Olson *et al.*, 1965; Fernández-Puentes y Carrasco, 1980; Olmo *et al.*, 2001). Esta diferencia se ha explicado por la permeabilidad alterada de estas células junto con la habilidad de las ribotoxinas para interaccionar con membranas que contengan fosfolípidos ácidos (Gasset *et al.*, 1989,1990; Martínez-Ruiz *et al.*, 2001; Olmo *et al.*, 2001).

La α-sarcina, la restrictocina y Asp f 1 son las ribotoxinas más exhaustivamente estudiadas (Arruda *et al.*, 1992; Gasset *et al.*, 1994; Wool, 1997; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001; García-Ortega *et al.*, 2005a), pero se han identificado y caracterizado parcialmente muchas otras, de distintas especies fúngicas (Lin *et al.*, 1995; Parente *et al.*, 1996; Huang *et al.*, 1997; Wirth *et al.*, 1997; Martínez-Ruiz *et al.*, 1999a,b). Estos estudios sugieren que entre las ribotoxinas existe un elevado grado de conservación, pues todas las estudiadas muestran secuencias de aminoácidos con similitudes de más del 85% (Figura 2). La única excepción conocida sería la hirsutelina A (HtA), otra proteína ribonucleolítica extracelular producida por el hongo patógeno de invertebrados *Hirsutella thompsonii* que ha sido incluida recientemente en la familia de las ribotoxinas (Herrero-Galán *et al.*, 2008b) y sólo muestra alrededor de un 25% de identidad de secuencia con los miembros de la familia descritos anteriormente (Boucias *et al.*, 1998; Martínez-Ruiz *et al.*, 1999a; Herrero-Galán *et al.*, 2008b). Según esto, parece que la presencia de las ribotoxinas entre los hongos está más extendida de lo que inicialmente se consideró (Martínez-Ruiz *et al.*, 1999b). Por ejemplo, se ha descrito que una RNasa específica purificada de las semillas maduras de una cupresácea oriental (*Biota orientalis*) también rompe un único enlace fosfodiéster del rRNA 28S en ribosomas de rata, pero en una región distinta al SRL, aunque cercana a ella espacialmente (Xu *et al.*, 2004).

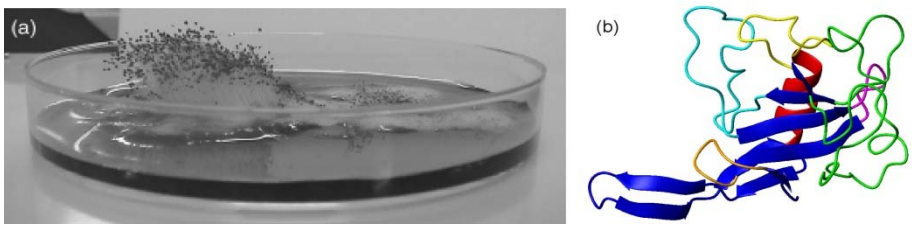

Figura 1. (a) Fotografía de un cultivo de *Aspergillus giganteus* MDH 18894. (b) Estructura tridimensional de la α-sarcina (PDB ID 1DE3). El diagrama se generó con el programa MOLMOL (Koradi *et al.*, 1996).

El género *Aspergillus* comprende un grupo complejo y ubicuo de hongos filamentosos con más de 185 especies, incluidas 20 patógenos humanos así como otras usadas para la producción industrial de alimentos y enzimas. La publicación de la secuencia genómica del organismo modelo *Aspergillus nidulans* (Galagan *et al.*, 2005) ha creado grandes expectativas en la búsqueda de avances en el conocimiento de la biología de estos microorganismos. También se ha publicado un estudio genómico comparativo, incluyendo dos especies más, *Aspergillus fumigatus* y *Aspergillus oryzae* (Machida *et al.*, 2005; Nierman *et al.*, 2005). *Aspergillus nidulans* no produce ninguna ribotoxina, mientras que *A. fumigatus*, un patógeno importante de humanos, produce Asp f 1, una de las ribotoxinas mejor conocidas (Moser *et al.*, 1992). *Aspergillus oryzae* (Machida *et al.*, 2005) se utiliza en la producción de sake, miso y salsa de soja, y también de RNasa T1 (Sato y Egami, 1957), una de las proteínas más exhaustivamente caracterizadas. La RNasa T1 es de hecho el miembro mejor conocido de la familia de RNasas extracelulares fúngicas (Yoshida, 2001; Loverix y Steyaert, 2001), un grupo que, obviamente, incluye a las ribotoxinas. Todas ellas muestran un elevado grado de similitud

de secuencia (Sato y Uchida, 1975a; Sacco *et al.*, 1983; Martínez-Ruiz *et al.*, 1999a,b) y estructura tridimensional (Pace *et al.*, 1991; Noguchi *et al.*, 1995; Yang y Moffat, 1996; Campos-Olivas *et al.*, 1996a,b; Pérez-Cañadillas *et al.*, 2000) (Figura 2) pero, aparte de las ribotoxinas, no se ha descrito que ninguna de estas RNasas extracelulares tenga actividad citotóxica.

Otra proteína a destacar es la RNasa U2, de *Ustilago sphaerogena* (Figura 2) (Arima *et al.*, 1968a,b; Sato y Uchida, 1975a). Se trata de la RNasa extracelular microbiana no tóxica más cercana a las ribotoxinas desde un punto de vista filogenético (Sacco *et al.*, 1983; Martínez del Pozo *et al.*, 1988; Martínez-Ruiz *et al.*, 1999a,b,2001). La RNasa U2 es una proteína pequeña y extremadamente ácida que muestra una fuerte preferencia por el enlace fosfodiéster en posición 3' de nucleótidos de purina (Rushizky *et al.*, 1970; Uchida *et al.*, 1970), algo bastante inusual en el grupo de RNasas microbianas. La RNasa T1, por ejemplo, presenta una especificidad estricta por el grupo guanilo. Ambas enzimas también difieren en sus valores de pH óptimo, que es ácido para la RNasa U2 y neutro para la RNasa T1, pero las dos son enzimas ciclantes, que rompen el RNA en dos pasos independientes, transfosforilación e hidrólisis (Yasuda y Inoue, 1982).

Las ribotoxinas son proteínas más grandes, generalmente básicas, que contienen bucles más largos y cargados que no están presentes en las RNasas fúngicas no tóxicas (Figura 2), lo que sugiere que estos bucles son la base estructural de su toxicidad (Martínez del Pozo *et al.*, 1988). Es posible que en algún momento una RNasa similar a la T1 adquiriera especificidad por el ribosoma mediante la inserción de pequeños dominios de reconocimiento que la habrían llevado hacia sustratos más específicos. Esto hace muy interesante el estudio de la evolución y el mecanismo de específicos. Esto hace muy interesante el estudio de la evolución y el mecanismo de acción de las ribotoxinas, pues parecen ser toxinas diseñadas con un objetivo, evolucionadas a partir de las otras RNasas microbianas no tóxicas para entrar en las células e inactivar específicamente los ribosomas (Lamy *et al.*, 1992; Kao y Davies, 1995). Identificar las características estructurales que han permitido a estas proteínas llegar a ser toxinas tan eficientes podría ser un paso decisivo hacia la utilización de las ribotoxinas, nativas o modificadas, como armas contra distintas patologías humanas.

Se ha especulado durante mucho tiempo sobre el mecanismo desarrollado por el hongo productor para superar la toxicidad de las ribotoxinas, pues sus propios ribosomas son susceptibles de ser atacados (Miller y Bodley, 1988). No hay evidencias de que se produzca simultáneamente ninguna antitoxina o proteína inhibidora que pueda bloquear la actividad citotóxica antes de su secreción al medio extracelular (Martínez-Ruiz *et al.*, 1998b) como ocurre con algunas toxinas ribonucleolíticas bacterianas (Muñoz-Gómez *et al.*, 2005; Condon, 2006; Kamphuis *et al.*, 2006; Luna-Chávez *et al.*, 2006). Las ribotoxinas son sintetizadas como precursores que maduran en compartimentos de membrana celular (Endo *et al.*, 1993a,b). Además, la caracterización de la pro-α-sarcina, producida por *Pichia pastoris*, reveló que es ribonucleolíticamente activa (Martínez-Ruiz *et al.*, 1998). En consecuencia, los datos acumulados hasta ahora sugieren que la protección de las células productoras frente a los efectos tóxicos de las ribotoxinas se basa en un reconocimiento eficiente de sus secuencias señal, seguido de una adecuada compartimentación antes de ser secretadas al medio extracelular.

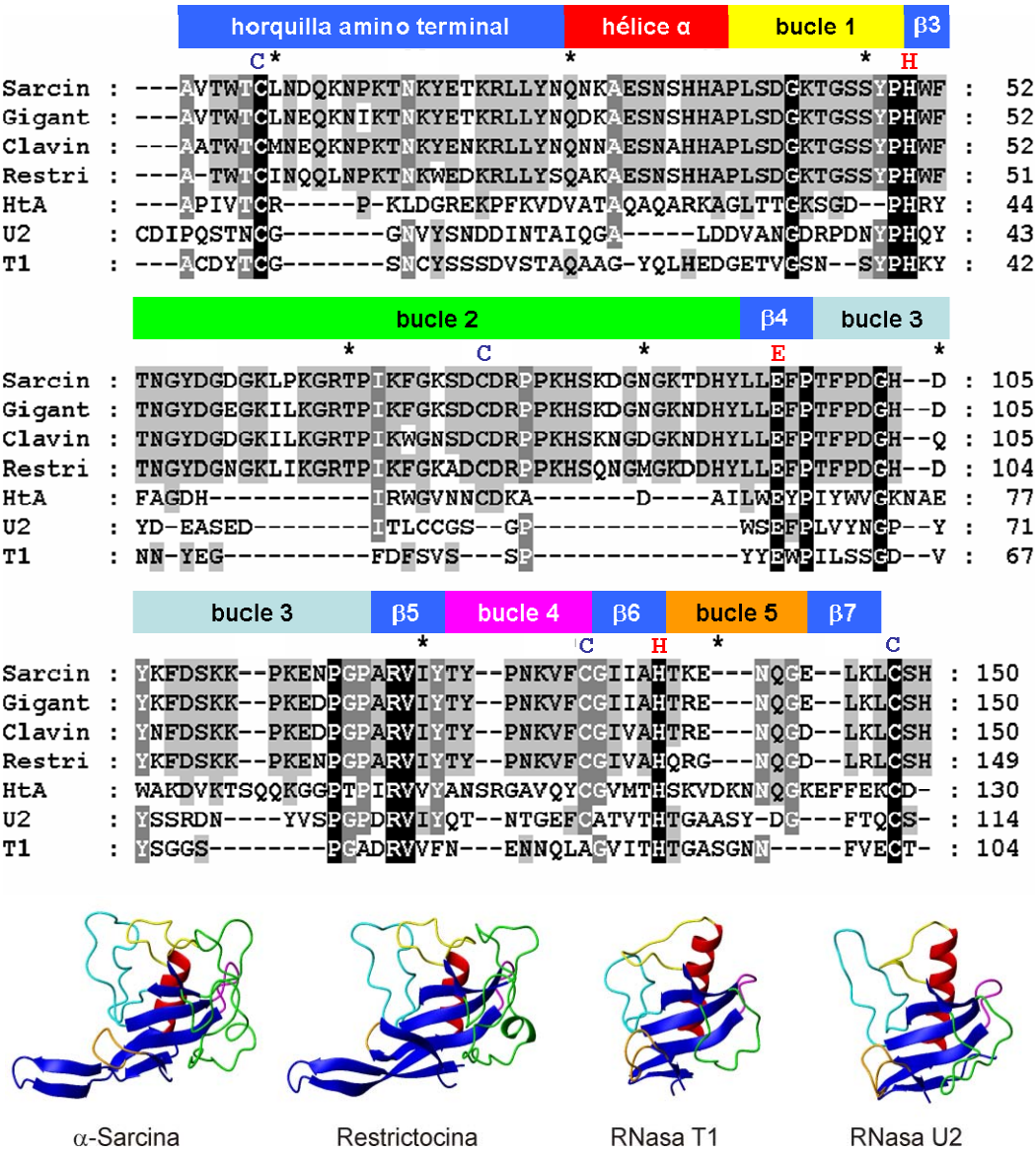

Figura 2. Alineamiento de secuencia de varias ribotoxinas (α-sarcina, gigantina, clavina, restrictocina y HtA) y de las RNasas T1 y U2. Los residuos de cisteína de las ribotoxinas se indican en azul y los tres residuos catalíticos de todas las proteínas en rojo. También se muestran las estructuras tridimensionales de la α-sarcina, la restrictocina y las RNasas T1 y U2. Las estructuras tridimensionales de las proteínas se ajustaron con las coordenadas atómicas de los residuos del centro activo (α-sarcina: 48, 50, 96, 121, 137, 145; restrictocina: 47, 49, 95, 120, 136, 144; RNasa T1: 38, 40, 66, 77, 92, 100; RNasa U2: 39, 41, 62, 85, 101, 110) y con los puentes disulfuro comunes a las cuatro proteínas (α-sarcina, 6-148; restrictocina, 5-147; RNasa T1, 6-103; RNasa U2, 9-113) (raíz cuadrada media de la desviación del ajuste, 1.877). Los diagramas y ajustes se generaron con el programa MOLMOL (Koradi *et al.*, 1996).

Las proteínas inactivadoras del ribosoma (RIPs) son otro grupo de proteínas tóxicas altamente especializadas, producidas por plantas y hongos (Stirpe *et al.*, 1988,1992; Nielsen y Boston, 2001) que actúan en la misma región del rRNA que las ribotoxinas (Schindler y Davies, 1977; Endo y Wool, 1982; Endo *et al.*, 1987; Correll *et al.*, 1998,1999; Mears *et al.*, 2002). Desde este punto de vista, se podría considerar que las ribotoxinas están incluidas entre las RIPs, pero algunos autores (Nielsen y Boston, 2001; Peumans *et al.*, 2001) prefieren que el nombre RIP se restrinja a las N-glicosidasas de plantas, representadas por la ricina, que despurinan un nucleótido contiguo al enlace fosfodiéster que hidrolizan las ribotoxinas (Endo *et al.*, 1987; Endo y Tsurugi, 1987). Por estas razones, la secuencia conservada del rRNA diana de las ribotoxinas y las RIPs se conoce universalmente como lazo sarcina/ricina, o SRL (Figura 3).

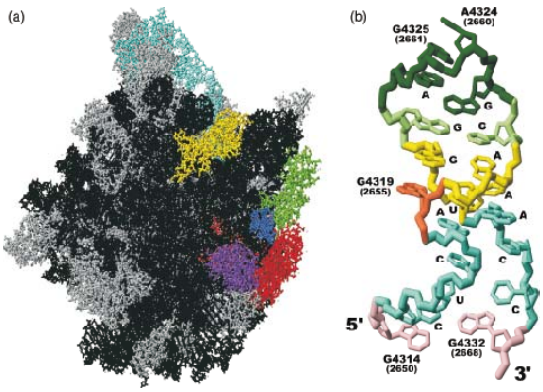

Figura 3. (a) Diagrama de la estructura de la subunidad mayor del ribosoma de *Halobacterium marismortui* (PDB ID 1JJ2): en negro, el RNA 23S; en cian, el RNA 5S; en azul, el SRL; en rojo, la proteína ribosomal L3; en verde, la proteína ribosomal L6; en amarillo, la proteína ribosomal 10e; en púrpura, la proteína ribosomal L14; y en gris, otras proteínas ribosomales. (b) Diagrama de la estructura del SRL (Correl *et al.*, 1998). La numeración corresponde las posiciones de los nucleótidos en el rRNA 28S (23S) de rata o de *Escherichia coli* (entre paréntesis). Las ribotoxinas rompen el enlace en el lado 3' de la G4325 (2661) (verde oscuro). La ricina despurina la A4324 (2660) (verde oscuro). La G prominente es la G4319 (2655) (naranja). Se han coloreado la región Watson-Crick de la horquilla (violeta), la región flexible (cian), el cruce de la hebra que incluye a la G prominente (amarillo) y el tetrabucle (verde). Los diagramas se generaron con el programa MOLMOL (Koradi *et al.*, 1996)

En el genoma de muchos procariotas aparecen codificadas tanto una toxina estable como una antitoxina lábil bajo el control de un operón simple. Estos sistemas toxina-antitoxina (TA) constituyen otra interesante familia de endo-RNasas tóxicas (Christensen *et al.*, 2003; Muñoz-Gómez *et al.*, 2005; Condon, 2006; Kamphuis *et al.*, 2006; Luna-Chávez *et al.*, 2006). Normalmente la antitoxina se sintetiza en cantidades equimoleculares a la toxina, y así se inhibe la acción ribonucleolítica, pero cuando las células se encuentran bajo algunas condiciones de estrés, la antitoxina se inactiva y la acción de la toxina provoca una parada del crecimiento celular en espera de condiciones más favorables. La diana de estas proteínas no está del todo establecida, pero los datos publicados hasta ahora apuntan al mRNA como mejor candidato. No puede descartarse, en

cambio, que el ribosoma o, más probablemente, el complejo translacional, ejerza algún tipo de estimulación o actividad moduladora sobre los sistemas TA (Christensen y Gerdes, 2003), aspecto que parece haber sido recientemente confirmado para las toxinas RelE y Kid (Diago-Navarro *et al.*, 2008). Así, estas endo-RNasas también pueden considerarse como moduladores de la biosíntesis de proteínas y en este sentido presentan una conexión funcional con las ribotoxinas fúngicas. De hecho, comparten con ellas el mecanismo de hidrólisis, pues también se comportan como RNasas ciclantes (véase más adelante). Sin embargo, aquí terminan las similitudes, pues es sabido que los procariotas no producen ribotoxinas, que sus toxinas utilizan un par de cadenas laterales de aminoácidos diferentes para la catálisis ácido-base, y que hasta ahora no se ha descrito la producción de antitoxinas específicas de ninguna ribotoxina (Martínez-Ruiz *et al.*, 1998,2001; Kamphuis *et al.*, 2006).

Características estructurales

Durante casi dos décadas se han determinado las secuencias completas o parciales de varias ribotoxinas (Rodríguez *et al.*, 1982; Sacco *et al.*, 1983; López-Otín *et al.*, 1984; Fernández-Luna *et al.*, 1985; Arruda *et al.*, 1990; Wirth *et al.*, 1997; Martínez-Ruiz *et al.*, 1999a,b) comprobándose que todas muestran un elevado grado de identidad en su secuencia de 150 aminoácidos (Figura 2), incluida la conservación de sus dos puentes disulfuro (Martínez del Pozo *et al.*, 1988; Martínez-Ruiz *et al.*, 2001). Las diferencias entre ellas se concentran principalmente en los bucles (Martínez-Ruiz *et al.*, 1999a). Estas observaciones incluyen también a la HtA (Martínez-Ruiz *et al.*, 1999a), a pesar de ser 20 residuos más corta que el resto de las ribotoxinas conocidas (Figura 2).

La similitud de secuencia también se manifiesta en la estructura tridimensional de las tres ribotoxinas estudiadas a ese nivel, la restrictocina (Yang y Moffat, 1996; Yang *et al.*, 2001), la α-sarcina (Pérez-Cañadillas *et al.*, 2000,2002; García-Mayoral *et al.*, 2005a,b) y la hirsutelina (Viegas *et al.*, 2009). La resonancia magnética nuclear (RMN) y otras técnicas han permitido además construir un mapa muy detallado de las propiedades estructurales y dinámicas de la segunda de ellas (Campos-Olivas *et al.*, 1996a,b; Pérez-Cañadillas *et al.*, 2000,2002; García-Mayoral *et al.*, 2005a,b). En cuanto a la estructura tridimensional (Figura 1), la α-sarcina se pliega en una estructura α+β con una lámina β central antiparalela de 5 hebras y una hélice α de casi tres giros. La lámina se compone de las hebras β3, β4, β5, β6 y β7, con una topología -1, -1, -1, -1 (Figuras 1 y 2) (Campos-Olivas *et al.*, 1996a,b; Pérez-Cañadillas *et al.*, 2000). Está muy alabeada, definiendo una cara convexa contra la que la hélice α se empaqueta ortogonalmente, y una superficie cóncava donde se alojan los residuos del centro activo: His 50, Glu 96, Arg 121 e His 137, con sus cadenas laterales proyectándose hacia fuera de la cavidad (Figura 4). Por otra parte, los residuos 1-26 forman una gran horquilla β que puede considerarse como dos pequeñas subhorquillas β consecutivas conectadas por una región bisagra. La primera está más cerca del extremo abierto de la horquilla, mientras que la segunda subhorquilla está formada por dos pequeñas hebras β1b y β2b conectadas por un giro β de tipo I.

Esta última parte de la horquilla amino terminal sobresale de la estructura, quedando como una protuberancia muy expuesta al disolvente, un detalle que es importante para su función, como se explica más adelante. Los demás tramos de su secuencia forman largos bucles que conectan los elementos de estructura secundaria (Figura 1). A pesar del carácter expuesto de esos bucles y de su falta de estructura secundaria ordenada, su conformación está bien definida, manteniéndose por redes de interacciones entre átomos de un mismo o de distinto bucle, que incluyen enlaces de hidrógeno, interacciones hidrofóbicas y puentes salinos (Yang y Moffat, 1996; Pérez-Cañadillas *et al.*, 2000). Desde un punto de vista dinámico, las medidas de RMN han mostrado que la α-sarcina se comporta como un rotor con simetría axial de tipo prolato, y que está compuesta por un núcleo hidrofóbico rígido y algunos segmentos expuestos, principalmente los bucles, que experimentan movimientos internos rápidos (de picosegundos a nanosegundos) (Pérez-Cañadillas *et al.*, 2002). La α-sarcina y la restrictocina muestran estructuras prácticamente idénticas (Figura 2), pero se observan algunas pequeñas diferencias concernientes a los largos bucles de estructura aperiódica y especialmente a la horquilla β arriba mencionada, una región cuya gran movilidad (Pérez-Cañadillas *et al.*, 2002) impidió que apareciese en la estructura cristalina de la restrictocina (Yang y Moffat, 1996).

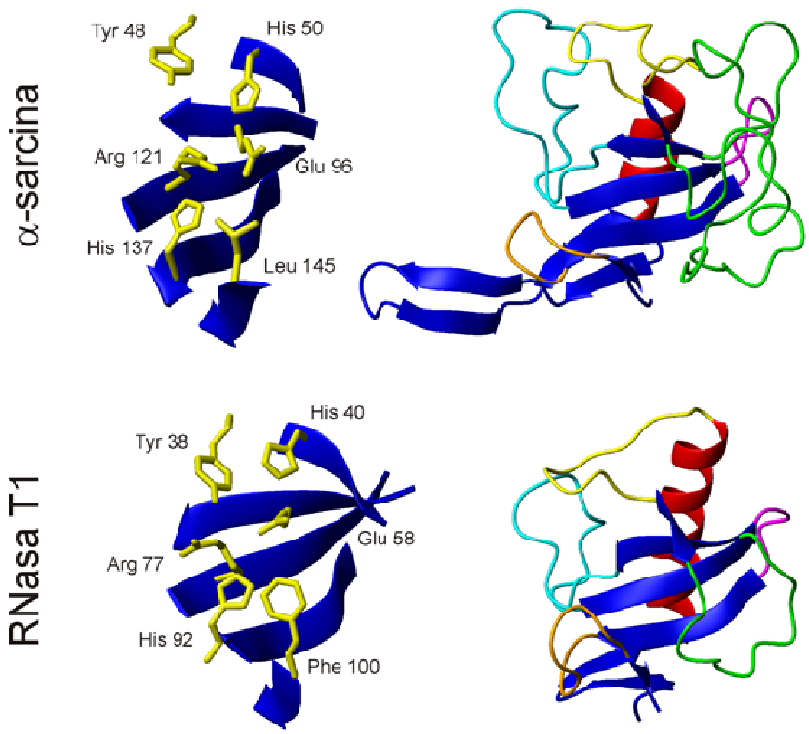

Figura 4. Representación de la disposición geométrica de las cadenas laterales de residuos localizados en el centro activo de la α-sarcina y la RNasa T1. Sólo se muestran las cadenas laterales de los residuos catalíticos implicados directamente en el mecanismo de corte general ácido-base. También se muestran las estructuras tridimensionales de ambas proteínas. Los diagramas se generaron con el programa MOLMOL (Koradi *et al.*, 1996).

Las ribotoxinas comparten su núcleo estructural con las RNasas de la familia de la T1, hecho que concuerda con la similitud de secuencia observada (Figuras 2 y 4). Por ejemplo, la comparación de las estructuras tridimensionales de la α-sarcina y la restrictocina con la de las RNasas T1 y U2 revela que las cuatro proteínas comparten idénticos elementos de estructura secundaria ordenada, así como la disposición de los residuos implicados en el centro activo, a pesar de las diferencias en sus secuencias de aminoácidos (Figuras 2 y 4). Así, todas las RNasas fúngicas extracelulares cuya estructura tridimensional se conoce muestran bastantes diferencias en la especificidad enzimática, pero comparten ese plegamiento estructural común que concierne a la arquitectura y conectividad de los elementos de estructura secundaria (Yang y Moffat, 1996; Campos-Olivas *et al.*, 1996b; Pérez-Cañadillas *et al.*, 2000; Martínez-Ruiz *et al.*, 2001). Las diferencias estructurales más significativas entre ellas están, de nuevo, relacionadas tanto con la presencia de una gran horquilla β amino-terminal en las ribotoxinas como con las diferencias de longitud y carga de sus bucles de estructura aperiódica (Figuras 2 y 4).

El bucle 2 de la α-sarcina (de la Thr 53 a la Tyr 93) (Figura 2), con una conformación bien definida, merece una mención especial por sus implicaciones funcionales (Pérez-Cañadillas *et al.*, 2000). Es altamente móvil, rico en glicocolas y residuos cargados positivamente, y está muy expuesto al disolvente. En este bucle, la secuencia comprendida entre los residuos 52 y 54 se encuentra inmovilizada en el armazón molecular (Pérez-Cañadillas *et al.*, 2002). La Asn 54 de esa secuencia es un residuo conservado entre las RNasas extracelulares fúngicas (Mancheño *et al.*, 1995a) que establece un enlace de hidrógeno entre el protón de su cadena lateral amida y el grupo carbonilo de la Ile 69, siendo esos protones muy resistentes al intercambio con el disolvente. Esta interacción también está conservada en el resto de RNasas de la familia de la T1 (Sevcik *et al.*, 1991; Pfeiffer *et al.*, 1997, Hebert *et al.*, 1998). Modelos de ajuste (*docking*) de la α-sarcina con el ribosoma también sugieren que el segmento formado por los residuos 51 a 55 de la proteína (Figura 2) podría interaccionar específicamente con el SRL en las proximidades del enlace a hidrolizar (Pérez-Cañadillas *et al.*, 2000), una predicción que parece confirmada por cristalografía de rayos X (Figura 5a) (Yang *et al.*, 2001). En esa interacción hay tres residuos de Lys del bucle 3 (Lys 111, Lys 112 y Lys 114) que parecen ser especialmente importantes, pues contactan con el elemento más identificativo del SRL, la G prominente (ver más adelante y Figura 5a) (Yang y Moffat, 1996; Pérez-Cañadillas *et al.*, 2000; Yang *et al.*, 2001).

Siguiendo con las zonas de interacción de la α-sarcina con su sustrato, el ribosoma, también debe destacarse la horquilla β amino-terminal. Un mutante en el que se eliminó la parte externa de esa horquilla, denominado α-sarcina Δ(7-22) (García-Ortega *et al.*, 2002), mantiene la misma conformación que la proteína silvestre, como se comprobó tras su caracterización espectroscópica (García-Ortega *et al.*, 2002) y la determinación de su estructura tridimensional en disolución por RMN (García-Mayoral *et al.*, 2004). Modelos predictivos *in silico* y análisis enzimáticos han revelado que esta parte expuesta de la horquilla β amino-terminal podría establecer interacciones con proteínas ribosomales específicas para así dirigir la ribotoxina a la región SRL del ribosoma (García-Ortega *et al.*, 2002; García-Mayoral *et al.*, 2005b) (Figura 5).

La α-sarcina es una proteína muy cargada, con un punto isoeléctrico elevado. El gran contenido en residuos cargados positivamente posiblemente es un requisito necesario no sólo para el reconocimiento y unión a su diana, muy cargada negativamente, el rRNA, sino también a las membranas celulares. Además, contiene ocho residuos de tirosina y dos de triptófano, que han sido estudiados espectroscópicamente. Inicialmente, usando medidas de absorbancia UV, emisión de fluorescencia y dicroísmo circular (CD), se describieron cinco transiciones conformacionales inducidas por pH, correspondientes a valores de pKa de 2.5, 4.5, 8.0, 10.2 y 11.4 (Martínez del Pozo *et al.*, 1988). Los dos últimos (10.2 y 11.4) corresponden a dos poblaciones diferentes de Tyr con distinta accesibilidad al disolvente. La transición a pH 8.0 se asignó al grupo amino en posición α del residuo amino terminal; los residuos de Asp y Glu se desprotonan a pH 4.5; y los valores de pKa de 2.5 y 10.2 se consideraron transiciones de desnaturalización. Esta caracterización adquirió posteriormente mucho mayor detalle, cuando los valores de pKa de todos los residuos de ácido aspártico, ácido glutámico e histidina de la α-sarcina se determinaron por RMN. Entonces se encontró que muchos de esos valores de pKa están muy alterados, incluidos algunos de residuos del centro activo (Pérez-Cañadillas *et al.*, 1998). Mucho más recientemente también se han medido sistemáticamente los valores de pKa de todos los residuos susceptibles de ser titulados, o se han predicho, cuando la medida directa no ha sido posible por el desplegamiento de la proteína (García-Mayoral *et al.*, 2003). Estas medidas y predicciones se extendieron también a una serie de mutantes del centro activo (E96Q, H50Q, H137Q, y H50/137Q) (García-Mayoral *et al.*, 2003,2006). Esta caracterización tan detallada se completó determinando el estado tautomérico de todas las cadenas laterales de los residuos de histidina (Pérez-Cañadillas *et al.*, 2003).

En cuanto a los dos residuos de triptófano de la α-sarcina, en posiciones 4 y 51, se encuentran conservados en todas las ribotoxinas estudiadas hasta ahora (Figura 2). La caracterización de mutantes en los que uno o ambos se sustituyeron por Phe (los mutantes sencillos W4F y W51F, y el doble mutante W4/51F) mostró que no son esenciales para la actividad ribonucleolítica específica de la proteína, aunque la actividad de los mutantes del Trp 51 sí aparece disminuida (De Antonio *et al.*, 2000). Pero aún más importante es que con esos mutantes se descubrió que el Trp 51, que no es fluorescente, es responsable de la mayoría de la señal de dicroísmo circular en el UV próximo de la proteína y que contribuye a su elipticidad global en la región del enlace peptídico (De Antonio *et al.*, 2000).

Finalmente, también es importante desde un punto de vista estructural mencionar que el centro activo de la α-sarcina está formado por, al menos, los residuos His 50, Glu 96, His 137, Tyr 48, Leu 45 y Arg 121, aunque sólo los tres primeros están directamente implicados en los pasos de transferencia de protones en el mecanismo catalítico (Lacadena *et al.*, 1999; Martínez-Ruiz *et al.*, 2001). Como ya se ha mencionado, están localizados en la lámina β central, con sus cadenas laterales apuntando hacia la cara cóncava de la estructura de la proteína (Figura 4). Las características más representativas de este centro activo son: (1) una alta densidad de residuos cargados; (2) valores de pKa inusuales para His 50, Glu 96, e His 137; (3) formas tautoméricas Nδ inusuales en His 50 e His 137, característica común de las RNasas microbianas; (4) presencia de un enlace de hidrógeno estructuralmente importante entre la His 137 catalítica y un oxígeno del esqueleto en el bucle 5; y

(5) baja accesibilidad de superficie de todos los átomos susceptibles de ser titulados (Pérez-Cañadillas *et al.*, 1998,2000,2003).

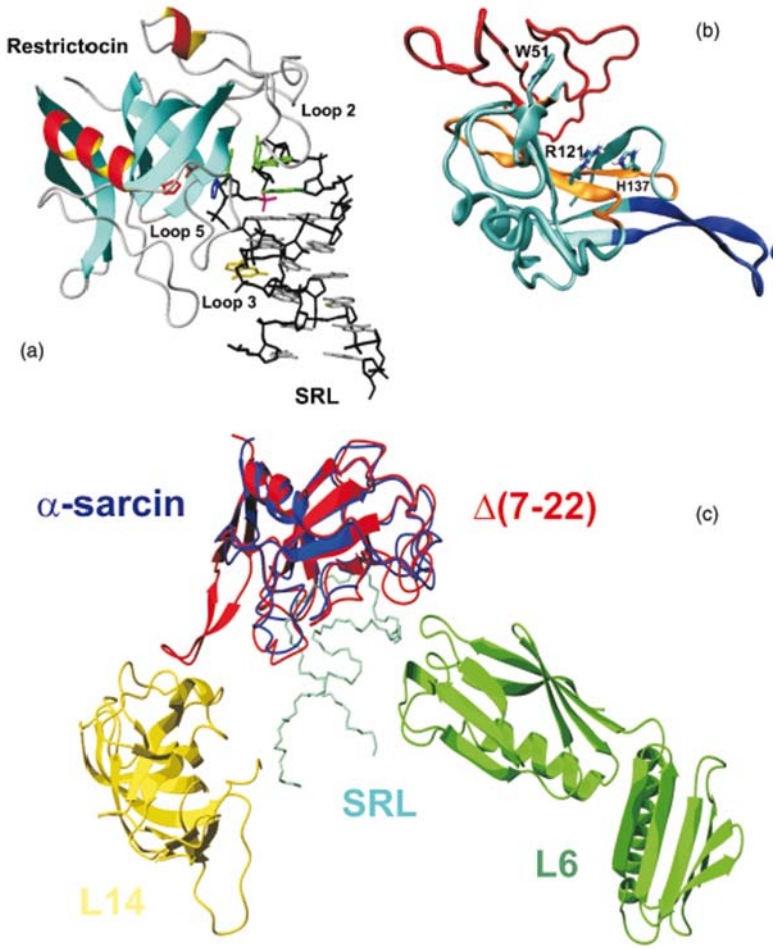

Figura 5. (a) Diagrama de la estructura cristalina de un complejo de la restrictocina con un análogo del SRL (Yang *et al.*, 2001) (PDB 1JBS). La estructura del análogo está distorsionada con respecto a la del SRL silvestre, y esto explica que no se produjera el corte, lo que permitió la cristalización del complejo. También se muestran las cadenas laterales de la His 49 (azul), el Glu 95 (rojo), y la His 136 (rojo). El esqueleto del RNA se muestra en negro, con las bases en gris excepto la G prominente, en amarillo, el tetrabucle en verde, y el grupo fosfato del enlace susceptible de ruptura en magenta. También se indican los bucles equivalentes a los bucles 2, 3 y 5 de la α-sarcina. (b) Diagrama de las regiones de la α-sarcina presumiblemente implicados en el establecimiento de interacciones con las bicapas de fosfolípidos: azul, residuos 7-22; naranja, residuos 116-139 y 51; rojo, residuos 53-93 (bucle 2). También se muestran las cadenas laterales de Trp 51, Arg 121 e His 137. (c) Modelo de ajuste reducido de la interacción de la α-sarcina silvestre (PDB ID 1DE3) y el mutante Δ(7-22) (PDB ID 1R4Y) con el SRL (PDB ID 430D) y las proteínas ribosomales L6 y L14 de *Halobacterium marismortui* (García-Mayoral *et al.*, 2005 a,b). Los diagramas se generaron con los programas MOLMOL (a, c) (Koradi *et al.*, 1996) y VMD (b) (Humphrey *et al.*, 1996).

Paso a través de membranas

La actividad antitumoral de la α-sarcina aparece cuando ejerce su actividad ribonucleolítica tras su paso selectivo a través de algunas membranas celulares. Así, aunque el SRL es una estructura universalmente conservada, la célula sólo resulta dañada si la ribotoxina ha sido capaz de atravesar la membrana plasmática para acceder a los ribosomas. Ya que hasta ahora no se han descrito receptores proteicos para la α-sarcina, su especificidad debe relacionarse con una interacción diferencial con los componentes lipídicos de las membranas. Hace tiempo se demostró que la α-sarcina es un potente inhibidor de la síntesis de proteínas en células infectadas por picornavirus (Fernández-Puentes y Carrasco, 1980), y que ionóforos (Alonso y Carrasco, 1981,1982), ATP externo (Otero y Carrasco, 1986) o un tratamiento con fosfolipasa C (Otero y Carrasco, 1986) convierten a las células de mamífero en diana de la α-sarcina. Todas estas observaciones se interpretaron considerando la existencia en todos esos casos de una permeabilidad de membrana alterada.

Usando sistemas modelo de lípidos se probó que la α-sarcina interacciona específicamente con vesículas de fosfolípidos que a pH neutro o ligeramente ácido están cargados negativamente, dando como resultado complejos proteína-lípido que pueden ser aislados por centrifugación en un gradiente de sacarosa (Gasset *et al.*, 1989). Experimentos de unión revelaron que la fuerte interacción entre la ribotoxina y los liposomas (K_d = 60.0 nM) causa la agregación de éstos, seguida por su fusión hasta estructuras lipídicas mucho mayores (Gasset *et al.*, 1989). La relación molar fosfatidilcolina/fosfatidilglicerol mínima requerida para este comportamiento es 1:10, y no es dependiente ni de la longitud ni del grado de insaturación de la cadena acilo del fosfolípido, siendo más efectivo a temperaturas inferiores a la temperatura de fusión del fosfolípido empleado. La saturación se alcanza a una relación molar lípido/proteína de 50:1, y el efecto es máximo a 0.15 M de fuerza iónica. En cambio, se abole a pH básico (Gasset *et al.*, 1990).

El paso inicial en la interacción entre la α-sarcina y las vesículas lipídicas es la formación de un dímero de vesículas mantenido por puentes proteína-proteína, según revelaron estudios cinéticos de dispersión de luz (*light-scattering*) empleando técnicas de flujo detenido (Mancheño *et al.*, 1994). Una vez que la agregación ha comenzado, ocurre la mezcla de lípidos entre las bicapas de las vesículas agregadas, de la misma forma que ocurriría con liposomas fusionándose. De hecho, esta fusión está provocada por el efecto desestabilizante de la proteína, que simultáneamente sufre cambios conformacionales hasta unirse a las vesículas (Mancheño *et al.*, 1994), como se vio por CD, emisión de fluorescencia y espectroscopía de infrarrojo ATR-FTIR (Gasset *et al.*, 1991b). Estos cambios conformacionales sugieren un incremento en el contenido en hélice α que, junto con los otros cambios espectroscópicos observados, se interpretó como un mecanismo mediante el cual los grupos polares de la proteína se protegen de los lípidos, lo que provocaría la formación de enlaces de hidrógeno intracatenarios y un menor apagamiento estructural (Gasset *et al.*, 1991b). De hecho, cuando la proteína se une a las vesículas, los enlaces peptídicos se protegen frente a la hidrólisis con tripsina (Gasset *et al.*, 1989; Oñaderra *et al.*, 1989) a pesar del gran número de residuos básicos

presentes en su secuencia (Sacco *et al.*, 1983). El efecto fusogénico atribuido a la α-sarcina fue corroborado por micrografías electrónicas de criofractura en las que, a la relación molar fosfolípido/proteína más alta (50:1), había una completa ausencia de estructuras vesiculares pequeñas, estando los lípidos, en cambio, organizados exclusivamente en láminas planas, indicando que los procesos de fusión habían tenido lugar de forma exhaustiva (Gasset *et al.*, 1990). Como paso final, y muy probablemente como consecuencia de la formación de estas grandes estructuras inestables, la α-sarcina también modifica la permeabilidad de las membranas, provocando la liberación de calceína atrapada en vesículas de fosfatidilglicerol (Gasset *et al.*, 1990). Medidas de polarización de fluorescencia, calorimetría diferencial de barrido y marcaje con fosfolípidos fotoactivos revelaron además que la α-sarcina, una proteína soluble en agua e hidrofílica, interacciona con bicapas fosfolipídicas a través de una combinación de fuerzas electrostáticas e hidrofóbicas (Gasset *et al.*, 1991a). De acuerdo con esto, la proteína sería inicialmente adsorbida a las cabezas polares y cargadas de los fosfolípidos, y entonces penetraría parcialmente en la interfase de la bicapa para interaccionar con una porción de las cadenas hidrocarbonadas de los lípidos (Gasset *et al.*, 1991a). El conjunto de todas estas observaciones es consistente con una intercalación de la proteína en la matriz lipídica, provocando la fusión y cambios de permeabilidad de las bicapas, procesos que presumiblemente están implicados en el paso de la proteína a través de la membrana de sus células diana ya que está descrito que en las membranas de células transformadas hay una mayor concentración de fosfolípidos cargados negativamente, como fosfatidilserina (Connor *et al.*, 1989; Gasset *et al.*, 1989, 1990; Zachowski, 1993). Desafortunadamente aún no hay evidencias directas de que esta abundancia de fosfolípidos ácidos sea lo que realmente explique la actividad antitumoral de la α-sarcina.

Corroborando esta teoría, dos tipos de ensayo demostraron la habilidad innata de la α-sarcina para atravesar membranas fosfolipídicas, si éstan son lo suficientemente ácidas, en ausencia de ninguna otra proteína (Oñaderra *et al.*, 1993). En el primero, la proteína se degradaba completamente cuando se añadía externamente a vesículas de asolectina que encapsulaban tripsina, un experimento llevado a cabo en presencia de tal cantidad de inhibidor de tripsina en el exterior de las vesículas que era imposible la degradación debida a trazas de proteasa liberadas de las vesículas. En el segundo, la α-sarcina añadida externamente era también capaz de romper, de forma dependiente de su concentración, moléculas encapsuladas de tRNA de levadura (Oñaderra *et al.*, 1993).

En cuanto a las regiones de la proteína implicadas en la interacción con lípidos, los primeros indicios se obtuvieron usando péptidos sintéticos solubles correspondientes a secuencias de la lámina β central de la α-sarcina. Algunos de esos péptidos, uno de ellos de sólo 9 aminoácidos, son capaces de mimetizar, al menos de forma cualitativa, los efectos producidos por la proteína completa sobre vesículas de fosfolípidos ácidos, indicando que probablemente esa región de la proteína (residuos 116-139) está implicada en la interacción con las membranas celulares (Mancheño *et al.*, 1995b, 1998a). Estas conclusiones son compatibles con la observación de que una forma desnaturalizada de la α-sarcina que contiene hebras β como único elemento de estructura secundaria ordenada provoca la desestabilización del núcleo hidrofóbico de las bicapas (Gasset *et al.*,

1995). Usando los mutantes de Trp mencionados antes, también se demostró que ni el Trp 4 ni el Trp 51 se requieren para la interacción de la α-sarcina con membranas lipídicas (De Antonio *et al.*, 2000). En cambio, esta interacción provoca un gran incremento en el rendimiento cuántico del Trp 51, situado en la lámina β de la proteína (Figura 5b), viéndose su emisión de fluorescencia simultáneamente apagada por el antraceno incorporado a la región hidrofóbica de esas bicapas. Por otra parte, un estudio con mutantes que afectan a la Arg 121 del centro activo de la α-sarcina (R121K y R121Q), también localizado en la lámina β central (Figuras 4 y 5b), mostró que la pérdida de carga positiva en esa posición supone un enorme impedimento para que la proteína interaccione con membranas fosfolipídicas (Masip *et al.*, 2001). Estos resultados llevaron a formular la propuesta de que las proteínas que han evolucionado para interaccionar con RNA, como las ribotoxinas, habrían desarrollado determinantes estructurales y químicos para reconocer entramados de polifosfatos que podrían permitir el reconocimiento de una bicapa de fosfolípidos (Masip *et al.*, 2001). Resulta muy interesante que cuando se resolvió la estructura cristalina de la restrictocina, el residuo equivalente a la Arg 121 (la Arg 120) se encontraba unido por un enlace de hidrógeno a una molécula de fosfato cocristalizada en el centro activo (Yang y Moffat, 1996). En resumen, todos estos resultados indican que la lámina β central de la α-sarcina, que se predecía como una de las regiones menos apolares de la proteína (Martínez del Pozo *et al.*, 1988; Mancheño *et al.*, 1995b), está de hecho localizada en el núcleo hidrofóbico de la bicapa tras la interacción proteína-vesícula (Figura 5b) (De Antonio *et al.*, 2000).

Aparte de este núcleo hidrofóbico de la α-sarcina, mutaciones puntuales que afectan a residuos localizados en la horquilla β amino-terminal (Lys 11 y Thr 20) y la variante de deleción Δ(7-22) sugirieron que esta porción de la proteína también podría estar implicada en la interacción con membranas celulares (García-Ortega *et al.*, 2001,2002), pues presentan un patrón de interacción con vesículas lipídicas diferente al de la proteína silvestre. Cuando es la restrictocina la proteína ensayada, el comportamiento también es diferente al de la α-sarcina nativa (García-Ortega *et al.*, 2001). Debe mencionarse que las secuencias de la α-sarcina y la restrictocina difieren sólo en 20 aminoácidos, y que seis de esos cambios se concentran en la horquilla β amino-terminal (Figura 2). De acuerdo con esta idea, el comportamiento del mutante α-sarcina Δ(7-22) es perfectamente compatible con la ausencia de una región de la proteína capaz de interaccionar con vesículas (García-Ortega *et al.*, 2002).

Finalmente, el bucle 2 ha sido también propuesto por varios autores (Yang y Moffat, 1996; Martínez del Pozo *et al.*, 1988; Kao y Davies, 1999; Pérez-Cañadillas *et al.*, 2000) como una de las regiones de la proteína implicadas en la interacción con lípidos (Figura 5b). Las diferencias entre la estructura refinada por RMN de este bucle en la α-sarcina y la restrictocina (García-Mayoral *et al.*, 2005a,b) podrían ayudar a explicar su distinto comportamiento al atravesar membranas celulares, pero esta posibilidad aún no ha sido directamente estudiada.

Propiedades enzimáticas

Tras el descubrimiento de las ribotoxinas, su actividad enzimática permaneció desconocida durante mucho tiempo (Lamy *et al.*, 1992) hasta que en 1977 Schindler y Davies publicaron que la α-sarcina es capaz de inactivar tanto los ribosomas de *Saccharomyces cerevisiae* como los de *Escherichia coli*, aunque con eficiencias diferentes (Schindler y Davies, 1977). Sorprendentemente, ninguno de estos organismos, ni las células HeLa, eran susceptibles a la toxicidad de la α-sarcina cuando las células están intactas, sugiriendo que son refractarias a la entrada de la proteína. Un estudio más detallado concluyó que la acción de la α-sarcina sobre ribosomas purificados afecta a la translocación y a la hidrólisis de GTP catalizada por el factor de elongación EF-2. Finalmente, mediante electroforesis en gel se separaron las distintas especies de rRNA de los ribosomas de levadura tras la incubación con α-sarcina, observándose la aparición de un fragmento extra de unos 300 nucleótidos (el denominado fragmento α) correspondiente al extremo 3' del rRNA 28S (Figura 6a) (Schindler y Davies, 1977). Experimentos posteriores mostraron que la α-sarcina rompe el esqueleto fosfodiéster en el lado 3' de la G2661 (numeración de *E. coli*) (Endo y Wool, 1982), mientras que la ricina despurina el enlace N-glicosídico entre la ribosa y la base nitrogenada correspondiente al nucleótido adyacente a la A2660 por el lado 5' (posiciones que corresponden a la G4325 y la A4324 en el rRNA 28S) (Figura 3) (Endo y Tsurugi, 1987; Endo *et al.*, 1987).

Por tanto, las ribotoxinas son RNasas altamente específicas frente a ribosomas intactos y conservan esta especificidad cuando se ensayan frente a un rRNA desnudo que contiene al SRL. Sin embargo, si se emplean concentraciones elevadas también puede causar una digestión progresiva de todo el rRNA 28S sin que aparezca el fragmento α (Endo *et al.*, 1993a,b; Wool, 1996,1997). Incluso se ha observado que cuando se ensayan relaciones enzima/sustrato elevadas la α-sarcina digiere DNA (Wool, 1984; Endo *et al.*, 1993a,b). Esta actividad inespecífica ha sido aprovechada en algunos otros ensayos ribonucleolíticos mucho menos específicos, que no se basan en seguir la liberación del fragmento α. En estos ensayos, la falta de significado biológico (por la falta de especificidad y por las concentraciones requeridas, mucho mayores que las catalíticas) se compensa por una cuantificación de los resultados mucho más sencilla, así como por la posibilidad de analizar los productos o incluso los intermedios de la reacción. Así, aunque son menos específicos, estos ensayos han contribuido significativamente al estudio detallado del mecanismo de corte de las ribotoxinas (Lacadena *et al.*, 1994,1998; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001).

Normalmente se emplean cuatro tipos de ensayos enzimáticos en el estudio de las ribotoxinas (Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). El primero y más específico es uno que usa su sustrato natural: ribosomas purificados o, al menos, un lisado de reticulocitos libre de células (Kao *et al.*, 2001). La actividad específica puede visualizarse detectando la liberación de un fragmento α de 300-400 nucleótidos (dependiendo del origen de los ribosomas) en un gel de agarosa desnaturalizante teñido con bromuro de etidio (Figura 6a). La sensibilidad de este ensayo ha mejorado recientemente con la posibilidad de detectar este fragmento α por hibridación con una sonda específica de DNA marcada con fósforo ^{32}P (Korennykh *et al.*, 2006).

En orden decreciente de complejidad, y por tanto de especificidad, un segundo ensayo usado frecuentemente se basa en el empleo de cortos oligorribonucleótidos sintéticos que mimetizan la secuencia y estructura del SRL (oligos tipo SRL). Las ribotoxinas rompen estos oligos tipo SRL de forma específica, produciendo sólo dos fragmentos, que pueden separarse en un gel de poliacrilamida (Figura 6b) (Endo *et al.*, 1988; Wool *et al.*, 1992), aunque esta ruptura es varios órdenes de magnitud menos eficiente que la producida en ribosomas intactos (Glück y Wool, 1996; Wool, 1997).

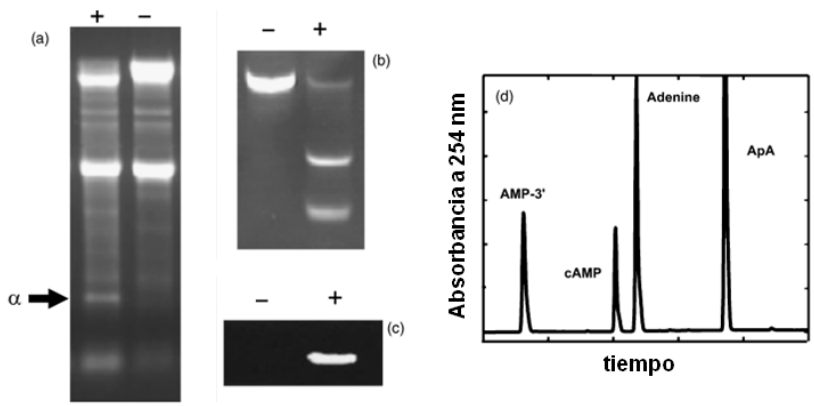

Figura 6. Ejemplos de los distintos ensayos utilizados para estudiar la actividad ribonucleolítica de las ribotoxinas. Se indica la presencia (+) o ausencia (-) de α-sarcina en el ensayo. (a) Ruptura específica de ribosomas de conejo. El fragmento α está indicado por una flecha. (b) Ruptura específica de un oligonucleótido de 35-mer que mimetiza el SRL. (c) Zimograma frente a poli(A). (d) Resolución por HPLC de los productos obtenidos tras la incubación de α-sarcina con ApA.

El tercer ensayo, mucho menos específico, es el zimograma (Figura 6c), en el que se evalúa la actividad ribonucleolítica frente a un homopolímero, como poli(A) o poli(I), embebido en un gel de poliacrilamida, tras la separación de las proteínas con una electroforesis en gel de poliacrilamida en presencia de SDS (PAGE-SDS) y su conveniente renaturalización eliminando el detergente. En algunos casos, este tipo de ensayo puede también llevarse a cabo en disolución, usando dispositivos de ultrafiltración para fraccionar los pequeños oligonucleótidos producidos en la reacción ribonucleolítica (Kao *et al.*, 2001). Una ventaja del zimograma es su uso adicional como control de homogeneidad de la muestra de proteína en cuanto a la ausencia de otras actividades ribonucleolíticas contaminantes.

El cuarto ensayo se basa en el hecho de que las ribotoxinas son también capaces de hidrolizar distintos dinucleósidos (o dinucleótidos) fosfato, como ApA (o ApAp), aunque con mucha menor eficiencia (Lacadena *et al.*, 1998). Este tipo de sustrato debe ser considerado como el que contiene los elementos mínimos para ser roto por una RNasa. La ventaja en este caso es que los productos, sustratos e intermedios de la reacción pueden ser separados y cuantificados por HPLC (Figura 6d), suministrando información sobre las distintas etapas (Lacadena *et al.*, 1998).

La combinación de estos diferentes ensayos y la producción y caracterización de muchos mutantes diseñados tanto aleatoriamente como de forma dirigida (Yang y Kenealy, 1992a,b; Lacadena *et al.*, 1995,1999; Kao *et al.*, 1998) han permitido no sólo la identificación de los residuos de las ribotoxinas implicados en la reacción catalítica, sino también la determinación de sus distintas funciones durante la ruptura del enlace fosfodiéster. Las RNasas no citotóxicas T1 y U2 han sido de gran ayuda en estos estudios como modelos de referencia. El mecanismo enzimático de la RNasa T1, por ejemplo, está perfectamente establecido (Figura 7), con la mayoría de los residuos que forman el centro activo asignados (Steyaert, 1997; Loverix y Steyaert, 2001; Yoshida, 2001). Esta enzima sigue el mecanismo general ácido-base de ruptura endonucleolítica de RNA en dos etapas. Primero, hay una reacción de transfosforilación para formar un intermedio, fosfato cíclico-2',3'. Segundo, este intermedio de reacción es hidrolizado al correspondiente fosfato-3' (Figura 7). La aparición de este intermedio cíclico, común a todas las RNasas de la familia de la T1 estudiadas hasta ahora, incluyendo a la RNasa U2, hace que estas enzimas se denominen RNasas ciclantes. El análisis de las reacciones de ruptura desarrolladas por la α-sarcina frente a distintos dinucleósidos monofosfato probó que también es una RNasa ciclante (Lacadena *et al.*, 1998,1999), con un pH óptimo de 5.0 (Pérez-Cañadillas *et al.*, 1998; Lacadena *et al.*, 1999). Por tanto, las ribotoxinas siguen el mismo esquema general de reacción que los otros miembros de la familia de la RNasa T1 aunque la eficiencia catalítica de las RNasas T1 y U2 frente a RNA desnudo, homopolinucleótidos o dinucleótidos es varios órdenes de magnitud mayor. En cambio, cuando se ensayan frente a sustratos naturales, las ribotoxinas rompen y consecuentemente inactivan el ribosoma con una constante de velocidad de segundo orden (k_{cat}/K_m de 1.7 x $10^{10} M^{-1} s^{-1}$) que iguala a la eficiencia catalítica de las enzimas más rápidas conocidas (Korennykh *et al.*, 2006).

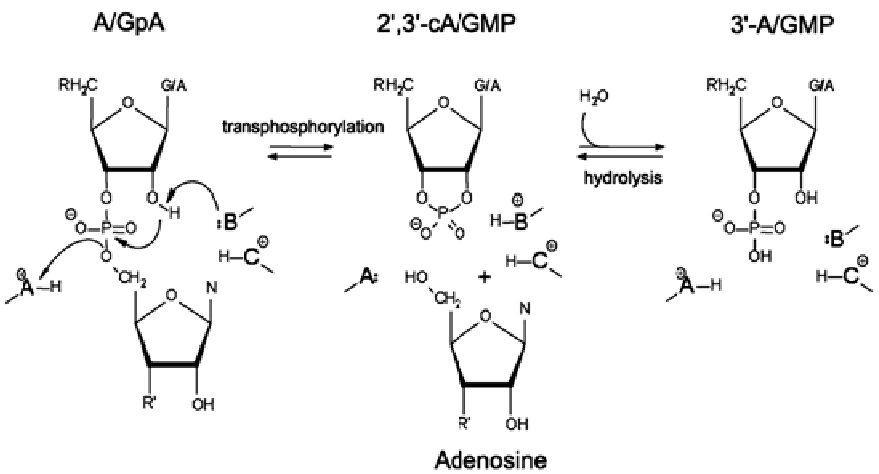

Figura 7. Modelo propuesto para el mecanismo catalítico de las RNasas ciclantes frente a sustratos dinucleósido (ApA o GpA). Un proceso de transfosforilación (en el que se producen el correspondiente mononucleótido cíclico 2'-3' y adenosina) va seguido por la hidrólisis del nucleótido cíclico, para producir el correspondiente mononucleótido-3'. (A), (B) y (C) son His 92, Glu 58 e His 40 en la RNasa T1 (Steyaert, 1997), e His 137, Glu 96 e His 50 en la α-sarcina (Lacadena *et al.*, 1999).

En el caso de la RNasa T1, durante el primer paso de la reacción, el Glu 58 actúa como una base general y la His 92, como un ácido general (Figuras 4 y 7). La hidrólisis del derivado cíclico está catalizada por los mismos grupos, pero con sus papeles intercambiados (Steyaert, 1997). De hecho, el par de residuos catalíticos más comúnmente encontrados en RNasas microbianas es esta combinación Glu/His (Yoshida, 2001). Otro residuo de histidina, la His 40, se requiere en su forma protonada para asistir a la estabilización electrostática del estado de transición y, si es necesario, parece ser capaz de adoptar la función de base general, como se vio con las variantes de la RNasa T1 con el Glu 58 mutado (Steyaert *et al.*, 1990; Steyaert, 1997). La superposición de las estructuras tridimensionales de las RNasas T1 y U2 con las de la α-sarcina y la restrictocina mostraron que los aminoácidos equivalentes eran la His 137, el Glu 96 y la His 50 en la α-sarcina, y la His 136, el Glu 95 y la His 49 en la restrictocina (Figura 4) (Sacco *et al.*, 1983; Martínez del Pozo *et al.*, 1988; Campos-Olivas *et al.*, 1996b). Además, cuando se intentó producir la restrictocina silvestre y varios mutantes en *S. cerevisiae* se observó que sólo era capaz de crecer la cepa que producía el mutante H136L (Yang y Kenealy, 1992a,b). Los mismos autores produjeron y caracterizaron parcialmente este mismo mutante en *A. niger* y *A. nidulans* (Brandhorst *et al.*, 1994) con resultados muy similares. No mucho después, otras variantes equivalentes de la α-sarcina (H137Q) y la restrictocina (H136Y) fueron aisladas y caracterizadas en detalle desde puntos de vista estructurales y funcionales; lo que confirmó que su falta de toxicidad se debía a la ausencia de actividad ribonucleolítica y no a cambios conformacionales importantes (Kao y Davies, 1995; Lacadena *et al.*, 1995). Gracias a la producción y posterior caracterización de más mutantes que afectaban a esos tres residuos, en la α-sarcina y la restrictocina, ahora es bien conocido que la His 137 y el Glu 96 son los únicos residuos de la α-sarcina esenciales para llevar a cabo la reacción de tipo ácido-base (Brandhorst *et al.*, 1994; Kao y Davies, 1995,1999; Lacadena *et al.*, 1995,1999; Sylvester *et al.*, 1997; Kao *et al.*, 1998). La His 50 contribuiría a la estabilización del estado de transición, como la His 40 de la RNasa T1, pero en este caso no sería capaz de sustituir al Glu 96 como base general en el mutante E96Q (Figura 7) (Lacadena *et al.*, 1999). Esto se dedujo porque la sustitución de la His 50 (o la His 49 en la restrictocina) por distintos aminoácidos no inactiva completamente a la enzima, pero disminuye bastante sus valores de k_{cat}, mostrando solo actividades residuales enzimáticas o citotóxicas, dependiendo de la naturaleza del ensayo empleado (Nayak y Batra, 1997; Sylvester *et al.*, 1997; Lacadena *et al.*, 1999). Además, se probó que los tres residuos mencionados se requieren para la inactivación específica de los ribosomas, pues cada mutante individual, así como los mutantes dobles y el triple, carecen de esta actividad tan particular (Lacadena *et al.*, 1999).

El perfil de dependencia de la actividad de la α-sarcina frente al pH es el típico para una catálisis ácido-base, pero sensiblemente distinto de los descritos para las RNasas T1 y U2 (Arima *et al.*, 1968a,b; Sylvester *et al.*, 1997; Pérez-Cañadillas *et al.*, 1998; Lacadena *et al.*, 1999). El mutante H50Q de la α-sarcina también muestra un comportamiento bastante diferente, debido probablemente a la ausencia de la carga positiva en el entorno del Glu 96. Finalmente, mientras que la α-sarcina muestra una baja eficiencia para hidrolizar el intermedio cíclico, como hacen la mayoría de las RNasas ciclantes, su variante H50Q es mucho más eficiente produciendo el AMP-3' a pH 7.0 (Lacadena *et al.*, 1999). Las medidas de RMN mencionadas antes también se usaron para calcular cómo estos residuos del centro activo muestran valores de pKa lejanos a sus valores intrínsecos, lo

que explicaría los distintos comportamientos no sólo en términos de especificidad sino también de dependencia con el pH (Pérez-Cañadillas *et al.*, 1998; Lacadena *et al.*, 1999).

En el cristal del complejo de la RNasa T1 con el sustrato mínimo 3'-GMP (Loverix y Steyaert, 2001), la Tyr 38, la Arg 77 y la Phe 100 también aparecen formando parte del sitio catalítico de la enzima. Se ha especulado que esos tres residuos, junto con la His 40, formarían un microambiente dieléctrico y estructural complementario en forma y carga al estado de transición y que además tendría capacidad para formar enlaces de hidrógeno con los oxígenos ecuatoriales de este estado de transición, contribuyendo a su solvatación/desolvatación óptima (Loverix y Steyaert, 2001). La Tyr 48, la Arg 121 y la Leu 45 son sus tres correspondientes estructurales en la α-sarcina (Figuras 2 y 4), y por tanto también han sido estudiados.

La Arg 77 de la RNasa T1 se localiza en las proximidades del grupo fosfato del sustrato, pero su posible papel funcional no se conoce, pues todos los intentos de aislar alguna RNasa T1 con una mutación que afecte a ese residuo han sido infructuosos (Steyaert, 1997). Así, se ha propuesto durante mucho tiempo que la Arg 77 de la RNasa T1 debía de facilitar el ataque nucleofílico, pero nunca se ha probado directamente por mutagénesis dirigida (Steyaert, 1997). Por otro lado, la Arg 121 de la α-sarcina se ha reemplazado por Gln o Lys, mutaciones que no modifican la conformación de la proteína, pero que abolen su actividad inactivadora de ribosomas. Sorprendentemente, estos mutantes aún son activos frente a sustratos pequeños e inespecíficos como ApA (con una K_m similar y una eficiencia catalítica menor a la de la proteína silvestre) (Masip *et al.*, 2001). Además, como se mencionaba antes, la pérdida de la carga positiva en esa posición produce cambios dramáticos en la habilidad de la α-sarcina para interaccionar con membranas fosfolipídicas (Masip *et al.*, 2001).

La Leu 145 de la α-sarcina ocupa la posición equivalente a la Phe 100 de la RNasa T1 (Figuras 2 y 4). La cadena lateral de la Phe 100 es un elemento catalítico apolar que estabiliza las separaciones de carga que se dan en el estado de transición, mediante el control del entorno dieléctrico (Doumen *et al.*, 1996). La caracterización de una variante de la α-sarcina, L145F, reveló que este mutante sigue siendo una RNasa activa (exhibe una K_m similar y una eficiencia catalítica ligeramente inferior frente a ApA), pero muestra una menor especificidad que la proteína silvestre frente a rRNA y a sustratos tipo-SRL (Masip *et al.*, 2003). La Leu 145 también se reveló como esencial para preservar el entorno electrostático del centro activo requerido para mantener el pKa anormalmente bajo descrito para la His 137 catalítica (Masip *et al.*, 2003).

En el mutante L145F de la α-sarcina se observó que la Asn 54 era uno de los residuos que mostraba un mayor desplazamiento en el espectro de RMN con respecto a la proteína silvestre. Se trata de un residuo conservado del bucle 2 (Figura 2) que no sólo contribuye a la estabilidad de las ribotoxinas, sino que además se requiere para su acción específica sobre los ribosomas, de acuerdo con los resultados obtenidos tras la caracterización de cinco mutantes distintos con cambios en esa posición (Siemer *et al.*, 2004). Estos resultados sugirieron que la Asn 54 está implicada en la disposición conformacional local del sitio de unión del sustrato. Las mutaciones en esa posición dan lugar a RNasas menos eficientes, especialmente frente a sustratos no específicos como poli(A). Normalmente, los residuos implicados en la interacción con la base en el lado 5' del enlace diana se

denominan residuos de reconocimiento. Los resultados obtenidos con los mutantes de la Asn 54 de la α-sarcina señalan que los residuos 53 a 56 (52 a 55 en la restrictocina) formarían ese bolsillo de reconocimiento en las ribotoxinas (Yang y Moffat, 1996). Sin embargo, el reconocimiento específico del ribosoma implica una red de interacciones mucho más compleja, en la que la mayoría no se ven perturbadas por la mutación de la Asn 54, lo que explicaría por qué casi todos los mutantes mantenían la capacidad de liberar específicamente el fragmento α (Siemer *et al.*, 2004). En conjunto, estos resultados concordaban perfectamente con la idea de los reordenamientos conformacionales locales de los mutantes del centro activo de la Asn 54 y la Leu 145, dando lugar a enzimas menos específicas y menos citotóxicas (Masip *et al.*, 2003; Siemer *et al.*, 2004). Otra observación importante de este trabajo fue comprobar que las ribotoxinas carecen de un residuo equivalente al Glu 46 de la RNasaT1, implicado en discriminar entre guanina y adenina (Gohda *et al.*, 1994). La falta de este residuo podría explicar por qué esta ribotoxina se limita a preferir purinas cuando se ensaya frente a RNA desnudo, y no es específica de guanina, como en el caso de la RNasa T1 (Endo *et al.*, 1983, 1988).

La Tyr 48 de la α-sarcina está conservada no sólo dentro de la familia de las ribotoxinas, sino también en todas las RNasas extracelulares fúngicas (Figura 2) (Martínez-Ruiz *et al.*, 1999a,b). Su equivalente, la Tyr 38 de la RNasa T1, forma un puente de hidrógeno con uno de los oxígenos del fosfato en el complejo RNasa T1/3'-GMP, interacción que debe de ser más favorable en el estado de transición (Loverix y Steyaert, 2001). El mutante Y48F de la α-sarcina resultó ser inactivo frente a sustratos de RNA polimérico, descubriéndose con este experimento el papel esencial del grupo OH en el anillo fenólico de la Tyr 48 (Álvarez-García *et al.*, 2006). Este mutante, en cambio, sí es activo frente a ApA, lo que revela que retiene la actividad ribonucleolítica a ese nivel. En resumen, el grupo OH eliminado sólo contribuye ligeramente a la eficiencia catalítica frente a ApA, pero es esencial para la actividad característica de las ribotoxinas (la degradación específica del rRNA y los sustratos tipo SRL).

Así, la Tyr 48, la Arg 121 y la Leu 145 parecen ser determinantes en la actividad ribotoxina de la α-sarcina. Al estudiar las estructuras cristalinas de complejos de la restrictocina con inhibidores se propuso que estas ribotoxinas debían usar un mecanismo que permitiese distorsionar la disposición de las bases nitrogenadas de los nucleótidos de su enlace diana para facilitar la ruptura del SRL en el sitio adecuado (Yang *et al.*, 2001). Todos los estudios llevados a cabo hasta ahora sugieren que estos tres residuos contribuirían a facilitar esta distorsión de las bases que permitiría al triplete His50/Glu96/His137 llevar a cabo la rotura de un único enlace fosfodiéster (Yang *et al.*, 2001).

Además, en estudios con distintos mutantes de la α-sarcina (García-Ortega *et al.*, 2001) y la mitogilina (otra ribotoxina que sólo se diferencia de la restrictocina en un residuo) (Kao y Davies, 1999;2000) se ha mostrado que la horquilla amino-terminal modula la actividad catalítica de las ribotoxinas. Estos estudios incluyeron variantes de deleción en los que esa horquilla había sido eliminada sin afectar a la estructura tridimensional global de la proteína (García-Ortega *et al.*, 2002,2005a; García-Mayoral *et al.*, 2004). Estos mutantes [α-sarcina Δ(7-22) y Asp f 1 Δ(7-22)]

conservan su actividad ribonucleolítica inespecífica así como su capacidad para romper específicamente oligonucleótidos tipo SRL, pero no son capaces de inactivar específicamente ribosomas de conejo, y por tanto son mucho menos citotóxicas (García-Ortega *et al.*, 2002,2005a).

Para concluir, es importante destacar las diferencias exhibidas por la HtA. Como era de esperar, ya que es una ribotoxina, esta proteína provoca una ruptura específica no sólo del rRNA 28S de conejo, sino también de los oligonucleótidos tipo-SRL usados como sustrato (Herrero-Galán *et al.*, 2008b). En cambio, cuando se emplean sustratos menos específicos, la HtA muestra un comportamiento bastante distinto, como refleja el hecho de que no es activa frente a poli(A), pero sí lo es frente a poli(C) (Herrero-Galán *et al.*, 2008b). Este comportamiento, que se ha observado en otras ribotoxinas silvestres y mutantes (Nayak *et al.*, 2001), debe ir unido a las diferencias estructurales que presenta la HtA, pero la interpretación no es obvia. Lo más probable es que este comportamiento refleje elementos del mecanismo catalítico aún desconocidos.

Interacción con el SRL y el ribosoma

Los ribosomas de los tres dominios filogenéticos, *Archea*, *Bacteria* y *Eukarya*, muestran diferencias en cuanto a sus componentes, pero algunas regiones funcionales están siempre conservadas, probablemente porque son esenciales para preservar la maquinaria de biosíntesis de proteínas (Mears *et al.*, 2002; Uchiumi *et al.*, 2002). Una de ellas es el SRL (Szewczak y Moore, 1995; Glück y Wool, 1996). Esta región es de particular interés por su papel crucial en los eventos relacionados con la elongación, tanto en ribosomas procariotas como eucariotas. Contiene una de las secuencias ribosomales más largas conservadas universalmente que se conocen (A2654–A2665 en el rRNA23S de *E. coli*, y A4318–A4329 en el rRNA 28S de rata), y muestra una conformación característica, también conservada estructuralmente. Está tan conservada que cuando se elucidó la estructura cristalina de la subunidad grande del ribosoma de *Halobacterium marismortui*, la secuencia del rRNA 23S se encajó en el mapa de densidad electrónica, nucleótido a nucleótido, empezando precisamente por la secuencia del SRL (Ban *et al.*, 2000). Este SRL es una horquilla distorsionada, con una parte central inusualmente rígida, y un tetrabucle GAGA, una G prominente en un cruce de la hebra, una región flexible, y un dúplex terminal de tipo A (Figura 3). No está asociado con ningún bolsillo de potencial electrostático intenso del ribosoma, pero junto con la región de unión a la L11, el tallo L7/L12, y las proteínas ribosomales L6 y L14 (Figuras 3 y 5c), constituye un sitio de unión de factores de elongación requerido para un correcto funcionamiento del ribosoma (Endo y Wool, 1982; Cameron *et al.*, 2002; Van Dyke *et al.*, 2002). La secuencia del dominio de unión de la proteína L11 está también universalmente conservada, de acuerdo con su papel esencial (Mears *et al.*, 2002). Curiosamente, la orientación espacial del SRL y el dominio de unión de la L11 en el ribosoma, región conocida como *centro GTPasa*, varía no sólo entre los distintos filos (Ramakrishnan y Moore, 2001; Mears *et al.*, 2002; Uchiumi *et al.*, 2002), sino también durante las distintas etapas de la formación del enlace peptídico (Gabashvili *et al.*, 2000). Estas

diferencias podrían explicar por qué distintas RIPs muestran afinidades distintas cuando se ensayan frente a ribosomas diferentes (Schindler y Davies, 1977; Endo y Wool, 1982; Wool *et al.*, 1992; Uchiumi *et al.*, 2002). Las mutaciones que afectan a la secuencia contenida en el SRL dan lugar a una unión deficiente de los factores de elongación y de los aminoacil-tRNA, así como a una menor fidelidad translacional (Liu y Liebman, 1996). Algunas de esas mutaciones son letales, reforzando la importancia de esta región para la maquinaria translacional (Leonov *et al.*, 2003). Estudios de la dinámica y la cinética del ribosoma muestran una movilidad considerable de ese centro GTPasa, y es posible que sufra cambios conformacionales esenciales para el correcto desarrollo de la traducción (Nilsson y Nissen, 2005).

Los estudios con oligonucleótidos sintéticos pequeños que mimetizan la secuencia del SRL (Correll *et al.*, 1998,1999,2003; Correll y Swinger, 2003) han vertido luz sobre los elementos del rRNA necesarios para que las ribotoxinas reconozcan el enlace fosfodiéster que va a ser hidrolizado. Estos análogos del SRL son de hecho reconocidos y rotos específicamente por las ribotoxinas (Endo *et al.*, 1988; Kao *et al.*, 2001), aunque, como se mencionó en el apartado anterior, se necesitan cantidades de enzima mayores, lo que indica que el reconocimiento no es tan específico como con el ribosoma completo. Incuestionablemente, han sido de gran ayuda, porque mantienen las características estructurales que posee el SRL en el ribosoma completo y se han usado para establecer cuáles son los determinantes necesarios para el reconocimiento del SRL por parte de las ribotoxinas. Así, se usaron modelos de ajuste (*docking*) y experimentos cinéticos para predecir las regiones del rRNA y de las proteínas ribosomales capaces de establecer interacciones con las ribotoxinas (Yang y Moffat 1996; Pérez-Cañadillas *et al.*, 2000; Correll *et al.*, 2004; García-Mayoral *et al.*, 2005b). Algunas de estas predicciones se confirmaron con la determinación de las estructuras cristalinas de complejos restrictocina-inhibidor hechos con distintas variantes de oligos de RNA tipo SRL (Figura 5a) (Yang *et al.*, 2001). Estos estudios incluían la resolución de las estructuras de dos versiones mutantes de oligonucleótidos que mimetizan el motivo SRL del rRNA 28S (Correll *et al.*, 2003), así como de otros tres análogos distintos del SRL acomplejados con la restrictocina (Yang *et al.*, 2001). Según los resultados obtenidos, hay dos áreas del SRL reconocidas tanto por las toxinas como por los factores de elongación: el tetrabucle GAGA y la G2655 prominente (Figura 3) (Moazed *et al.*, 1988; Glück y Wool, 1996; Munishkin y Wool, 1997; Pérez-Cañadillas *et al.*, 2000). La G2655 representa el sitio más crítico para la unión de los factores de elongación (Munishkin y Wool, 1997). En cambio, el tipo de nucleótido no parece ser determinante en el reconocimiento, sino más bien la conformación del SRL (Munishkin y Wool, 1997; Correll *et al.*, 1999,2003). La simulación dinámica molecular de dos estructuras del SRL basadas en estructuras cristalinas de motivos SRL de *E. coli* y rata revelaron que el tetrabucle GAGA es la parte más dinámica de este motivo (Špačková y Šponer, 2006). De hecho, los tetrabucles GNRA adoptan una geometría desplegada hasta el momento de la unión de los factores de elongación y/o las toxinas, como se observó en los complejos SRL-restrictocina que se han mencionado (Yang *et al.*, 2001). Ya se ha indicado que esos estudios llevaron a proponer que las ribotoxinas debían de usar un mecanismo de distorsión de la orientación de la base para facilitar la ruptura de los sustratos SRL en el sitio adecuado (Yang *et al.*, 2001), y que esa distorsión debía de ser un mecanismo común para todas las endonucleasas que actúan sobre sustratos plegados, como es el caso de las ribotoxinas (Yang *et al.*, 2001). En resumen, según los resultados mencionados

anteriormente, hay dos regiones distantes de las moléculas de ribotoxina que participan en su interacción específica con el SRL. Por un lado, la región rica en Lys correspondiente al bucle 3 de la α-sarcina interacciona con el enlace fosfodiéster cargado negativamente en los alrededores de la G prominente. Y, por otra parte, la secuencia dentro del bucle 2 que comprende los residuos 52-55 (una secuencia extendida que incluye a la ya mencionada Asn 54) y algunos residuos del bucle 5, contactan con el tetrabucle GAGA conservado que contiene el enlace que hidroliza la toxina (Figura 5a).

Es bastante obvio, sin embargo, que estas interacciones con el SRL no explican por sí solas la exquisita actividad específica mostrada por las ribotoxinas frente a los ribosomas intactos. Por tanto, se requieren otras interacciones con elementos ribosomales adicionales. En este sentido, se ha visto recientemente que el contexto ribosomal aumenta la velocidad de reacción en varios órdenes de magnitud. Esta ventaja catalítica parece surgir de las interacciones electrostáticas favorables con el ribosoma (Korennykh *et al.*, 2006). Las ribotoxinas cargadas positivamente se unen al ribosoma con gran afinidad y velocidad, aumentando así la velocidad de ruptura del SRL en varios órdenes de magnitud, igualando la eficiencia catalítica de las enzimas más rápidas conocidas (Korennykh *et al.*, 2006). La α-sarcina, por ejemplo, está muy cargada: el 39% de su superficie está compuesta por cadenas laterales cargadas y el 26% por cadenas laterales polares (Pérez-Cañadillas *et al.*, 2000). Por tanto, se sugiere un mecanismo de localización de la diana en el que la α-sarcina encuentra al ribosoma de forma aleatoria y entonces difunde por el campo electrostático ribosomal hasta el SRL. Hace tiempo se describió no sólo que la actividad ribonucleolítica de la α-sarcina se ve completamente inhibida por NH_4^+, K^+ o Na^+ en concentraciones superiores a 0.2 M, así como por niveles milimolares de algunos cationes divalentes como Ca^{2+}, Mn^{2+} o Mg^{2+} (Endo *et al.*, 1983; Martínez del Pozo *et al.*, 1989), sino también que une Zn^{2+}, Cd^{2+} y Co^{2+} con una afinidad correspondiente a constantes de disociación en el rango milimolar (Martínez del Pozo *et al.*, 1989). Se propuso que esta unión está mediada por interacciones con las cadenas laterales de His del centro activo, y que afecta a la emisión de fluorescencia, probablemente modificando al microambiente del Trp 51. En esos estudios se vio que los cationes Zn^{2+} son inhibidores efectivos de la actividad ribonucleolítica, mientras que la inhibición promovida por la mayoría de los otros cationes estudiados se debe al establecimiento de interacciones con los sustratos usados, más que a la existencia de interacciones específicas con la proteína (Martínez del Pozo *et al.*, 1989).

En este contexto, debe considerarse que hay movimientos internos que permiten a los elementos de reconocimiento del ribosoma sondear una parte significativa del espacio conformacional, incrementando las posibilidades de una unión exitosa. Como se ha explicado antes, los residuos 1-26 en la α-sarcina forman una gran horquilla β que puede ser considerada como dos láminas β consecutivas conectadas por una región bisagra. La segunda lámina β, que coincide con los residuos 7-22, es una de las regiones con mayor flexibilidad conformacional, apareciendo con un plegamiento independiente del núcleo de la proteína (Pérez-Cañadillas *et al.*, 2000,2002; García-Mayoral *et al.*, 2004). Los resultados obtenidos con el mutante α-sarcina Δ(7-22) mencionado sugirieron que la proteína interaccionaría con el ribosoma a través de, al menos, dos regiones, el bien conocido SRL del rRNA y una región diferente reconocida por la horquilla β de la proteína

(García-Ortega *et al.*, 2002). La estructura tridimensional del mutante en disolución (García-Mayoral *et al.*, 2004) mostró que conserva el plegamiento de la α-sarcina silvestre, incluida la conformación espacial de los bucles. Las diferencias más significativas se concentran en el bucle 2, la nueva orientación del bucle 3, y la dinámica del bucle 5, donde la heterogeneidad conformacional se observa como consecuencia de haber eliminado interacciones importantes con residuos del motivo original (García-Mayoral *et al.*, 2004). Además de su integridad estructural mantiene la habilidad para romper específicamente oligonucleótidos tipo SRL, pero desaparece el reconocimiento altamente específico de la α-sarcina por el ribosoma. Al modelar este reconocimiento de las ribotoxinas por el ribosoma usando las estructuras tridimensionales de las α-sarcinas silvestre y Δ(7-22) se propusieron dos interacciones más no identificadas hasta entonces (García-Mayoral *et al.*, 2005b). Una de ellas ocurriría entre una pequeña secuencia extendida del bucle 2 de la α-sarcina y la proteína ribosomal L6 (Figura 5c). La segunda ocurriría entre los residuos correspondientes a la parte distal delecionada de la horquilla β y la proteína L14 (Figura 5c). Estas dos proteínas ribosomales procariotas se localizan junto al SRL (Figura 3a), están presentes en los tres filos de organismos vivos (Ban *et al.*, 2000), y parecen sufrir los cambios más sustanciales durante el proceso de biosíntesis de proteínas, de acuerdo con los mapas del ribosoma de rayos X y criomicroscopía electrónica (Ban *et al.*, 2000; Gabashvili *et al.*, 2000). Obviamente, la interacción que implica a la horquilla β no es posible en el mutante de deleción y parece ser crucial para el reconocimiento específico del ribosoma (García-Mayoral *et al.*, 2005b). Esta hipótesis se vio reforzada con la observación de que una región homóloga a la secuencia 11-16 de la α-sarcina puede encontrarse en el EF-2 de *S. cerevisiae* (Kao y Davies, 1999; García-Mayoral *et al.*, 2005b). Tanto las ribotoxinas como las proteínas ribosomales L14 (o sus correspondientes L23 en organismos eucariotas) representan familias de proteínas altamente homólogas. Las regiones de interacción de la α-sarcina y la L14 están conservadas pero exhiben un cierto grado de variabilidad (García-Mayoral *et al.*, 2005b), especialmente considerando los residuos implicados de la L14 (las proteínas L23 tienen secuencias que se relacionan sólo lejanamente). Este hecho ayuda a explicar no sólo la extraordinaria eficiencia catalítica de las ribotoxinas frente a los ribosomas, sino también sus diferencias potenciales, dependiendo del origen del ribosoma ensayado (Schindler y Davies, 1977; Endo y Wool, 1982; Endo *et al.*, 1983; García-Mayoral *et al.*, 2005b).

Actividad de las ribotoxinas frente a células intactas

Ya se ha explicado que la α-sarcina es capaz de inactivar ribosomas en sistemas libres de células de una gran variedad de organismos (Endo *et al.*, 1993a,b; Kao y Davies, 1995), mientras que presenta una marcada selectividad cuando se usan células intactas como diana. Esta especificidad parece venir determinada por su habilidad para penetrar en las células. Así, la α-sarcina es activa frente a células de mamífero transformadas o infectadas por virus, en ausencia de ningún otro agente permeabilizante (Fernández-Puentes y Carrasco, 1980; Carrasco y Esteban 1982; Olmo *et al.*, 1993,2001; Turnay *et al.*, 1993; Stuart y Brown, 2006). La proteína también es citotóxica, inhibiendo

la biosíntesis de proteínas, cuando se ensaya frente a ocho líneas celulares tumorales humanas y de rata de origen mesenquimal, glial y epitelial (Turnay *et al.*, 1993). Este efecto se satura y es consistente con el hecho de que el paso a través de la membrana celular sea el paso limitante de velocidad pero, en cambio, no se detecta daño de esa membrana ni alteraciones de la actividad mitocondrial (Turnay *et al.*, 1993). De nuevo, estos experimentos confirmaron que la α-sarcina posee un carácter citotóxico intrínseco y bastante específico, en ausencia de ningún agente permeabilizante externo, virus incluidos, cuando se ensaya frente a algunas líneas celulares transformadas. Las razones particulares para esta selectividad a nivel molecular aún no se han establecido completamente; sin embargo, como se mencionaba antes, la presencia de fosfolípidos ácidos en la cara externa de la membrana parece ser uno de los factores determinantes (Connor *et al.*, 1989; Gasset *et al.*, 1989,1990; Zachowski, 1993).

Se ha estudiado el mecanismo de internación de la α-sarcina en células intactas de rabdomiosarcoma humano y los eventos que inducen la muerte celular (Olmo *et al.*, 2001). Según los resultados obtenidos, la toxina se internaliza vía endocitosis viajando en endosomas ácidos y a través del aparato de Golgi, como se dedujo del requerimiento de ATP y de los efectos del NH_4Cl, la monensina y la nigericina en su citotoxicidad. Además de la inhibición de la biosíntesis de proteínas asociada a la ruptura específica del rRNA 28S, la α-sarcina mata a las células de rabdomiosarcoma vía apoptosis. Mediante análisis comparativos de los efectos de la α-sarcina y la ciclohexamida se dedujo que esta apoptosis no es simplemente una consecuencia directa y general de la inhibición de la biosíntesis de proteínas (Olmo *et al.*, 2001), aunque experimentos con un mutante catalíticamente inactivo de la α-sarcina (H137Q), que no es citotóxico ni apoptótico, revelaron que esta muerte celular programada está directamente relacionada con los efectos catalíticos de la toxina sobre los ribosomas, pues ese mutante exhibe capacidades de interacción con lípidos idénticas a las de la proteína silvestre (Lacadena *et al.*, 1995).

La falta de carga positiva en la posición correspondiente a la Arg 121 de la α-sarcina produce un dramático impedimento de su capacidad para interaccionar con membranas fosfolipídicas (Masip *et al.*, 2001), apoyando la conclusión de que la Arg 121 es un residuo esencial para la citotoxicidad característica de la α-sarcina y, presumiblemente, de otras ribotoxinas fúngicas. De acuerdo con sus actividades ribonucleolíticas y de interacción con lípidos alteradas, todas las variantes estudiadas con mutaciones que afectan a la especificidad de la proteína, especialmente el mutante de deleción Δ(7-22), muestran efectos de citotoxicidad disminuida sobre células de rabdomiosarcoma (García-Ortega *et al.*, 2002). Incluso la restrictocina muestra una capacidad de interacción con fosfolípidos menor, lo que está correlacionado con una citotoxicidad disminuida (García-Ortega *et al.*, 2001), como ya se ha mencionado antes en esta revisión. Por otro lado, la HtA exhibe una citotoxicidad muy similar en términos de IC_{50} (concentración requerida para producir un 50% de inhibición de biosíntesis de proteínas) (Herrero-Galán *et al.*, 2008b).

Las ribotoxinas como alérgenos

Los hongos representan una de las principales fuentes de alérgenos. Dentro de ellos, *Aspergillus fumigatus*, un patógeno oportunista humano, ha sido bien estudiado (Walsh y Pizzo, 1988; Bodey y Vartivarian, 1989). Normalmente una infección invasiva de esta especie es fatal si no se trata pronto, e incluso entonces, la terapia antifúngica fracasa frecuentemente. La incidencia de infecciones por hongos ha aumentado últimamente, por el incremento en el número de pacientes inmunodeprimidos, y la infección por *A. fumigatus* es común en los postoperatorios (Pasqualotto, 2006). Entre las razones por las que *A. fumigatus* puede comportarse como un patógeno de humanos está la capacidad para crecer rápidamente a temperaturas tan altas como 50°C, siendo la especie más termófila de todas las de *Aspergillus* (Ronning *et al.*, 2005). El resto de los hongos productores de ribotoxinas crecen escasamente al cultivarlos por encima de los 30°C. También es remarcable que *A. fumigatus* es una fuente de alergia y asma más común que *A. nidulans* o *A. oryzae*, las otras dos especies de *Aspergillus spp.* cuyas secuencias genómicas han sido determinadas (Galagan *et al.*, 2005; Machida *et al.*, 2005; Nierman *et al.*, 2005). Curiosamente, todos los alérgenos de *A. fumigatus* tienen homólogos cercanos en las otras dos especies, con la excepción de la ribotoxina Asp f 1 y la metaloproteasa Asp f 5 (Ronning *et al.*, 2005), pero aún no está claro si Asp f 1 es un factor crítico en la provocación de una respuesta alérgica. En este aspecto, debe recordarse que los alérgenos normalmente se definen como aquellas proteínas reconocidas por anticuerpos IgE contenidos en el suero de los pacientes alérgicos. En relación con esto, es destacable que se encontró restrictocina en la orina de pacientes con aspergilosis diseminada (Arruda *et al.*, 1990,1992; Lamy *et al.*, 1991), así como acumulada en las proximidades de los nodos de la infección fúngica (Lamy *et al.*, 1991). Aunque se ha probado que Asp f 1 no es un factor de virulencia importante en las infecciones de *A. fumigatus* (Paris *et al.*, 1993; Smith *et al.*, 1993,1994), esta proteína está claramente implicada en la patogenicidad de la aspergilosis broncopulmonar alérgica (ABPA), la más severa de las enfermedades alérgicas por inhalación, pues estos pacientes presentan altos niveles de IgE específicos de Asp f 1 (Kurup *et al.*, 1994; García-Ortega *et al.*, 2005a). El ABPA tiene una prevalencia del 1-2% en pacientes con asma persistente, y este valor aumenta hasta el 15% en pacientes de fibrosis quística (Greenberger, 2002; Kurup *et al.*, 2006). La explicación de que *A. fumigatus* sea el hongo normalmente implicado en estas enfermedades parece ser, de nuevo, su ubicuidad y sus pequeñas esporas, que crecen de forma óptima a 37°C. De esta forma puede colonizar el tracto respiratorio del huésped, provocando el comienzo de eventos patológicos (Banerjee y Kurup, 2003).

En la diagnosis de reacciones alérgicas se usan con frecuencia extractos de *A. fumigatus*, pero se trata de mezclas complejas de hasta 200 proteínas, glicoproteínas y compuestos de bajo peso molecular (Piechura *et al.*, 1983) que son muy difíciles de estandarizar. Los intentos de mejorar la diagnosis se centran en el empleo de preparaciones estándar homogéneas de alérgenos producidos de forma recombinante (Crameri *et al.*, 1998; Kurup *et al.*, 2006). A este respecto es interesante destacar que Asp f 1 fue el primer alérgeno recombinante testado *in vivo* (Moser *et al.*,

1992), y que presentó una total concordancia con las determinaciones serológicas (Moser *et al.*, 1992; Crameri *et al.*, 1998; Hemmann *et al.*, 1999). Desgraciadamente el alérgeno nativo recombinante no está desprovisto de actividad citotóxica, y puede provocar anafilaxias.

Como se ha mencionado anteriormente, las ribotoxinas tienen bucles mucho más largos que las otras RNasas fúngicas no tóxicas, que presumiblemente están implicados en su especificidad, toxicidad y antigenicidad. De nuevo, debe tenerse en cuenta que la región 7-22, contenida en la horquilla β amino-terminal característica de las ribotoxinas, presenta la mayor variabilidad de secuencia de aminoácidos entre las ribotoxinas (Figura 3) (Martínez-Ruiz *et al.*, 1991a,b,2001), es altamente flexible y está muy expuesta al disolvente (Pérez-Cañadillas *et al.*, 2000; García-Mayoral *et al.*, 2004). De hecho, Asp f 1 difiere de la α-sarcina en sólo 19 aminoácidos (87% de identidad de secuencia), y cinco de esos 19 residuos se localizan en esa horquilla β amino-terminal. Como se asume generalmente que las regiones expuestas y flexibles de las proteínas son normalmente buenos candidatos a epítopos de células B, esta horquilla β podría ser un determinante principal de la inmunorreactividad de estas proteínas. Esto se confirmó con la producción y caracterización de Asp f 1, α-sarcina, y sus correspondientes variantes Δ(7-22) (García-Ortega *et al.*, 2002,2005a). En primer lugar, estos datos confirmaron la prevalencia significativa de anticuerpos IgE específicos de Asp f 1 en el suero de pacientes sensibilizados a *Aspergillus* (García-Ortega *et al.*, 2005a), como habían descrito anteriormente otros autores (Kao *et al.*, 2001; Greenberger, 2002; Kurup *et al.*, 2006). Este resultado fue particularmente importante en los pacientes de ABPA estudiados, pues en el 100% de ellos se encontraron anticuerpos IgE anti-Asp f 1. En segundo lugar, y aunque varios estudios preliminares con péptidos sintéticos que solapan con la región antes mencionada produjeron resultados controvertidos en cuanto a su comportamiento antigénico (Kurup *et al.*, 1998; Madan *et al.*, 2004), las tres proteínas estudiadas, Asp f 1 Δ(7-22), α-sarcina y α-sarcina Δ(7-22), presentaron marcados descensos en su reactividad frente a anticuerpos IgE anti-Asp f 1 (García-Ortega *et al.*, 2005a), indicando que la porción eliminada está implicada en, al menos, un epítopo alergénico. Sin embargo, aunque importante, éste no puede ser el único epítopo alergénico dentro de esta molécula, como se dedujo de experimentos de inhibición por ELISA (*enzyme linked immunosorbent assay*). Los residuos esenciales de los epítopos de Asp f 1 están cambiados en la α-sarcina silvestre, pues la respuesta frente al suero de los pacientes fue incluso menor para esta proteína que para el mutante Asp f 1 Δ(7-22). A pesar de su disminuida reactividad IgE, la prevalencia de las tres variantes de Asp f 1 se mantuvo prácticamente inalterada, y todas conservaron la mayoría de los epítopos IgG (García-Ortega *et al.*, 2005a). De esta forma, estas variantes de deleción no citotóxicas de las ribotoxinas son moléculas prometedoras para uso en la diagnosis y en terapias inmunomoduladoras en hipersensibilidad a *Aspergillus*. Sin embargo, aún se requieren ensayos *in vivo* para evaluar esa posibilidad.

Inmunotoxinas

Una de las estrategias de la terapia antitumoral es la preparación de inmunotoxinas, siguiendo la idea descrita por Ehrlich en 1956. Este premio Nobel fue quien presentó el concepto de convertir a las células tumorales en diana de "balas mágicas", que consisten en un transportador específico de tejido que distribuiría agentes tóxicos al tejido neoplásico (Ehrlich, 1956). En la pasada década se publicó una gran cantidad de investigación orientada a la clínica en relación a las inmunotoxinas (Reiter y Pastan, 1998; Kreitman *et al.*, 1999; Brinkmann, 2000; Li *et al.*, 2004). Las inmunotoxinas son agentes terapéuticos con un alto grado de especificidad, compuestas por un motivo que dirige a la molécula a su diana, como anticuerpos o ligandos fisiológicamente importantes (factores de crecimiento o citoquinas), unidos a una toxina, sobre todo proteínas tóxicas de plantas o bacterias (Brinkmann y Pastan, 1994; Reiter y Pastan, 1998; Kreitman, 2001).

Inicialmente, las inmunotoxinas se preparaban por conjugación de las toxinas a anticuerpos monoclonales. El motivo que dirigía a la inmunotoxina a su diana era la molécula de anticuerpo completa (Kreitman, 2000). Como los sitios de unión al antígeno están en las regiones variables de los anticuerpos, los estudios posteriores trataron de verificar que esos fragmentos Fab, obtenidos por digestión de las IgG con papaína, retenían la capacidad de interaccionar con antígenos (Ward *et al.*, 1989; Wörn y Plückthun, 2001), dando lugar a las llamadas inmunotoxinas Fab o Fv, que se internaban más fácilmente por su menor tamaño (Brinkmann, 2000). El desarrollo de tecnologías avanzadas ha permitido la producción de inmunotoxinas recombinantes, estabilizadas por un péptido flexible (scFv) o por un puente disulfuro entre los dominios variables (dsFv), con las ventajas de que pueden expresarse en varios organismos modelo, son fácilmente modificadas por ingeniería genética y son más estables (Kreitman, 2003; Li *et al.*, 2004).

En cuanto a la porción tóxica de la molécula, las toxinas más representativas empleadas han sido la ricina (de plantas) y la exotoxina A de *Pseudomonas* (PE) o la toxina de la difteria (DT) (de bacterias). La ricina está compuesta por dos subunidades unidas por un puente disulfuro, siendo la cadena A la responsable de la actividad glicosidasa que lleva a la inactivación de los ribosomas (Olsnes y Pihl, 1973a,b; Endo *et al.*, 1987), y la única que normalmente se usa para hacer inmunotoxinas (Ghetie *et al.*, 1993; Engert *et al.*, 1997; Schnell *et al.*, 1998). La ricina despurina un único nucleótido contiguo al enlace fosfodiéster que rompen las ribotoxinas (Endo y Tsurugi, 1987; Endo *et al.*, 1987), una actividad catalítica que también inactiva al ribosoma. Se han obtenido también diferentes inmunotoxinas que contienen la molécula de ricina completa o la cadena A desglicosilada (Pastan *et al.*, 1992; O'Toole *et al.*, 1998). En cuanto a las toxinas bacterianas, PE y DT son proteínas de cadena única que inhiben la síntesis de proteínas al ADP-ribosilar el EF-2 (Carroll y Collier, 1987). Entre las inmunotoxinas derivadas de PE y DT, las más comúnmente usadas implican versiones truncadas de las toxinas, producidas por escisión genética de su dominio de unión, dando como resultado las variantes PE38 o PE40 (Kondo *et al.*, 1988; Kreitman *et al.*, 1990,1993; Pastan, 2003), y las variantes DT388 o DT389 (Foss *et al.*, 1998; LeMaistre *et al.*, 1998), respectivamente.

Las ribotoxinas presentan varias ventajas para su uso en el diseño de inmunotoxinas, como su pequeño tamaño, su alta termoestabilidad, su resistencia a proteasas y su actividad ribonucleolítica tan eficiente (Gasset *et al.*, 1994; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). En cuanto a la restrictocina se han descrito, además, una baja inmunogenicidad y una menor toxicidad en ratón (Rathore y Batra, 1996). Así, se han usado distintas ribotoxinas como componentes de inmunotoxinas (Orlandi *et al.*, 1988; Conde *et al.*, 1989; Hertler y Frankel, 1989; Wawrzynczak *et al.*, 1991; Better *et al.*, 1992; Rathore y Batra, 1996;1997a,b; Rathore *et al.*, 1997). Inicialmente, las inmunotoxinas basadas en ribotoxinas se construían por conjugación química, como se describió para, entre otras, la mitogilina (Better *et al.*, 1992), la restrictocina (Orlandi *et al.*, 1988; Conde *et al.*, 1989; Rathore y Batra, 1996) y la α-sarcina (Wawrzynczak *et al.*, 1991). Las inmunotoxinas de segunda generación se diseñaron, principalmente con la restrictocina, por la fusión del cDNA de la restrictocina con el que codifica la región scFv del anticuerpo monoclonal dirigido contra el receptor de la transferrina humana (anti-TFR) (Rathore y Batra, 1997a,b), unidos por un péptido lineal flexible para promover el plegamiento independiente de las dos fracciones de la inmunotoxina. Estas construcciones se sometieron posteriormente a ingeniería para mejorar el procesamiento intracelular y la distribución de la restrictocina (Goyal y Batra, 2000).

Se han descrito algunas inmunotoxinas basadas en la α-sarcina (Wawrzynczak *et al.*, 1991; Rathore *et al.*, 1997), con la ribotoxina unida químicamente a anti-TFR o anti-Fib75. La α-sarcina usada en estas construcciones se obtuvo de cultivos de *A. giganteus* (Wawrzynczak *et al.*, 1991) o de la expresión heteróloga en *E. coli* (Goyal y Batra, 2000). Se obtuvieron resultados prometedores al medir la citotoxicidad, con valores de IC_{50} similares a los de inmunotoxinas basadas en toxinas bacterianas o de plantas (Goyal y Batra, 2000). La inmunotoxina con α-sarcina presentó una estabilidad equivalente a la de otras análogas, así como similares actividades farmacocinéticas y específica sobre las células diana (Wawrzynczak *et al.*, 1991). Sin embargo, no se han llevado a cabo estudios posteriores con inmunotoxinas basadas en la α-sarcina, incluyendo ensayos clínicos, probablemente por el gran tamaño de la molécula resultante, que podría dificultar la correcta internación en tumores sólidos, o por la baja estabilidad estructural de los inmunoconjugados. Pero debe tenerse en cuenta que estas inmunotoxinas de α-sarcina no se produjeron como inmunotoxinas de segunda generación recombinantes, como las inmunotoxinas de cadena simple (scFv-IMTX) descritas después para la restrictocina (Rathore y Batra, 1997a,b), que dieron mejores resultados en términos de estabilidad y ensayos de citotoxicidad *in vivo*. E incluso una scFv-IMTX podría ser modificada fácilmente por ingeniería genética para mejorar la actividad citotóxica o disminuir la toxicidad inespecífica *in vivo* o la inmunogenicidad.

En relación con esto, recientemente se ha producido una inmunotoxina de cadena única en la levadura metilotrófica *Pichia pastoris*, compuesta por el dominio variable del anticuerpo monoclonal B5, específico contra los carbohidratos de Lewis^{Y}, que son muy abundantes en carcinomas, unido a la α-sarcina a través de un péptido que contiene un sitio de ruptura por furina (scFv-IMTXαs) (Lacadena *et al.*, 2005). *P. pastoris* ha surgido como un sólido anfitrión de expresiones heterólogas, debido a la expresión secretora eficiente de proteínas recombinantes complejas con puentes disulfuro intermoleculares e intramoleculares correctos que no requiere estrategias *in vitro*

adicionales de desplegamiento y replegamiento, a diferencia de la mayoría de las inmunotoxinas expresadas de forma heteróloga en bacterias (Cregg *et al.*, 1993; Gurkan y Ellar, 2003,2005). De hecho, *P. pastoris* posee promotores regulados estrictamente, como el del gen de la alcohol oxidasa 1, que son perfectamente convenientes para la expresión controlada de genes exógenos (Cregg *et al.*, 1989). De esta forma se han producido con éxito varias inmunotoxinas extracelularmente en *P. pastoris* (Woo *et al.*, 2002,2004,2006; Lacadena *et al.*, 2005; Liu *et al.*, 2005).

El anticuerpo monoclonal (mAb) B5 pertenece a una familia de mAbs dirigidos contra antígenos relacionados con el carbohidrato de Lewis^{Y} que se sobreexpresa en la superficie de muchos carcinomas, incluidos los tumores sólidos de mama y colon (Pastan y Fitzgerald, 1991). Se han usado distintos miembros de la familia en muchas inmunotoxinas, como mAb B3 (Brinkmann *et al.*, 1991,1993; Pai *et al.*, 1991,1996; Benhar y Pastan, 1995a; Bera y Pastan, 1998), mAb B1 (Pastan y Fitzgerald, 1991; Benhar y Pastan, 1995b; Kuan y Pastan, 1996), y mAb B5 (Benhar y Pastan, 1995a,b). De hecho, mAb BR96 y vAb3S193 también se han evaluado para inmunoterapia dirigida (Trail *et al.*, 1993; Rosok *et al.*, 1998; Scott *et al.*, 2000). Al menos tres de estas inmunotoxinas o inmunoconjugados han sido evaluados recientemente en ensayos en fase I en pacientes con cáncer, con resultados prometedores (Pai *et al.*, 1996; Brinkmann, 2000).

La scFv-IMTXαs producida en *P. pastoris* presenta la actividad ribonucleolítica característica de la α-sarcina y una citotoxicidad específica frente a líneas celulares que contienen el antígeno de Lewis^{Y} (Lacadena *et al.*, 2005). Y se ha ido más allá, caracterizándose inmunotoxinas basadas en ésta y modificadas genéticamente para aumentar la afinidad y la estabilidad (Lacadena *et al.*, 2005).

Conclusiones y futuras perspectivas

Las ribotoxinas son RNasas únicas en cuanto a su especificidad y citotoxicidad. Su acción ribonucleolítica destacable y exquisitamente específica, así como su capacidad para atravesar membranas, han sido objeto de estudio durante muchos años, y ahora se conocen bien en términos moleculares, gracias a la combinación de una amplia variedad de técnicas estructurales, espectroscópicas, bioquímicas y celulares, junto con la producción y caracterización de un gran número de mutantes (Lamy *et al.*, 1992; Gasset *et al.*, 1994; Wool, 1997; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). La determinación de varias estructuras ribosomales de alta resolución y el uso de distintas vesículas lipídicas modelo y líneas celulares transformadas, susceptibles o no a la acción de estas toxinas, han sido de gran ayuda para descifrar los detalles del mecanismo citotóxico de las ribotoxinas a nivel molecular. También ha sido muy útil la existencia de RNasas extracelulares fúngicas no tóxicas bien conocidas, como la RNasa T1. Presumiblemente, estas RNasas no son tóxicas porque carecen de la capacidad antes mencionada de atravesar una bicapa fosfolipídica. Aún así han sido, y aún son, excelentes modelos de referencia con los que aproximarse al estudio de esta familia de proteínas. Desafortunadamente, la función natural de las ribotoxinas aún permanece

desconocida, siendo ésta, definitivamente, una de las cuestiones más interesantes que debe ser resuelta. En relación con esto aún se requieren estudios sobre la regulación de la producción de ribotoxinas en su ambiente natural. En realidad, hay muchas evidencias indirectas que sugieren que estas proteínas son sintetizadas bajo algunas condiciones de estrés (Olson *et al.*, 1965b; Meyer y Stahl, 2002,2003; Meyer *et al.*, 2002), pero aún se necesita una caracterización directa de estos mecanismos. A este respecto no debe descartarse la conexión funcional con los sistemas procariotas TA (Condon, 2006). En efecto, el descubrimiento y caracterización de la HtA, una ribotoxina singular desde puntos de vista estructurales y funcionales, ha abierto una nueva puerta a la adquisición de pistas adicionales sobre el origen y funcionalidad de las ribotoxinas fúngicas. La futura caracterización de esta proteína y otras similares, que probablemente aparecerán, dado el acelerado descubrimiento de nuevas ribotoxinas, mejorará enormemente nuestro conocimiento de la acción ribotóxica en su contexto natural.

Esta falta de conocimiento sobre la función natural, en cambio, no limita su empleo como agentes terapéuticos. A pesar de que su uso como agentes antitumorales se abandonó enseguida, debido a su alta toxicidad (Roga *et al.*, 1971), es cierto que la actual acumulación de datos sobre su mecanismo de acción permite la visión optimista de que estas ribotoxinas, o probablemente algunas variantes modificadas, puedan ser usadas con fines terapéuticos. En este sentido, la producción de mutantes hipoalergénicos e inmunotoxinas destacan como las alternativas más plausibles en el futuro a medio plazo.

En cuanto a la primera aproximación, debe remarcarse el posible empleo de *Lactococcus lactis*, que es un constituyente primario de muchos cultivos iniciadores industriales y artesanales usados para la producción de una amplia variedad de productos lácteos fermentados. En la actualidad, además, se han convertido en "fábricas celulares" en la producción de metabolitos y proteínas de membrana, y como un sistema de distribución de moléculas terapéuticas en el tracto gastrointestinal (Steidler *et al.*, 2000, Kunji *et al.*, 2005). El estatus de *L. lactis* como un organismo "generalmente considerado seguro" (GRAS, *generally regarded as safe*) confiere a este sistema las características requeridas para probar protocolos inmunoterapéuticos para enfermedades alérgicas relacionadas con Asp f 1. Con este objetivo en mente, se han obtenido cepas de *L. lactis* capaces de secretar las variantes hipoalergénicas de Asp f 1 mencionadas antes (Alegre-Cebollada *et al.*, 2005; García-Ortega *et al.*, 2005a), y aunque su uso como sistema potencial de distribución aún debe ser probado, constituyen una de las direcciones de investigación que deberían ser exploradas inmediatamente.

Las inmunotoxinas son otra alternativa prometedora para el empleo de las ribotoxinas como agentes terapéuticos contra procesos tumorales. La producción de grandes cantidades de versiones de inmunotoxina mejoradas de α-sarcina (Lacadena *et al.*, 2005) y otras RNasas microbianas ya está en marcha y es definitivamente una de las trayectorias de investigación a seguir en el futuro próximo.

Fungal ribotoxins: molecular dissection of a family of natural killers

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Aspf1; filamentous fungi; immunotoxin; RNase; sarcin.

Abstract

RNase T1 is the best known representative of a large family of ribonucleolytic proteins secreted by fungi, mostly *Aspergillus* and *Penicillium* species. Ribotoxins stand out among them by their cytotoxic character. They exert their toxic action by first entering the cells and then cleaving a single phosphodiester bond located within a universally conserved sequence of the large rRNA gene, known as the sarcin–ricin loop. This cleavage leads to inhibition of protein biosynthesis, followed by cellular death by apoptosis. Although no protein receptor has been found for ribotoxins, they preferentially kill cells showing altered membrane permeability, such as those that are infected with virus or transformed. Many steps of the cytotoxic process have been elucidated at the molecular level by means of a variety of methodological approaches and the construction and purification of different mutant versions of these ribotoxins. Ribotoxins have been used for the construction of immunotoxins, because of their cytotoxicity. Besides this activity, Aspf1, a ribotoxin produced by *Aspergillus fumigatus*, has been shown to be one of the major allergens involved in allergic aspergillosis-related pathologies. Protein engineering and peptide synthesis have been used in order to understand the basis of these pathogenic mechanisms as well as to produce hypoallergenic proteins with potential diagnostic and immunotherapeutic applications.

Introduction

Ribotoxins are a family of toxic extracellular fungal RNases that exert ribonucleolytic activity on the larger molecule of RNA in the ribosome, leading to protein synthesis inhibition and cell death by apoptosis (Gasset *et al.*, 1994; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). Several studies have suggested that their location on the surface of fungal conidiophores correlates with the maturation of the conidia (Brandhorst & Kenealy, 1992; Yang & Kenealy, 1992a, b). Ribotoxins were discovered during a screening program of the Michigan Department of Health, started in 1956, searching for antibiotics and antitumor agents. The culture filtrates of a mold isolated from a sample of Michigan farm soil were found to contain a substance inhibitory to both *sarcoma 180* and *carcinoma 755* induced in mice (Olson *et al.*, 1965b). The mold was identified as *Aspergillus giganteus* MDH18894 (Fig. 1a), and the molecule responsible for these effects proved to be a protein, named α-sarcin (Fig. 1b) (Olson & Goerner, 1965). Two other antitumor proteins, restrictocin and mitogillin, both produced by *A. restrictus*, were later found to have similar activities, and were therefore included within the same group of antitumor molecules. Aspf1, another ribotoxin, produced by *Aspergillus fumigatus*, was much later identified as a major allergen in *Aspergillus*-related diseases (Arruda *et al.*, 1992). Unfortunately, further studies revealed an unspecific cytotoxicity of these proteins, which limited their potential clinical uses (Roga *et al.*, 1971). The study of these toxins was abandoned until the mid-1970s, when it was demonstrated that they inhibited protein biosynthesis in ribosomal preparations at concentrations as low as 0.1 nM by specifically cleaving a single phosphodiester bond of the large rRNA gene fragment (Schindler & Davies, 1977; Endo & Wool, 1982). This bond is of particular interest, because it is located at an evolutionarily conserved site with important roles in ribosome function, elongation factor 1 (EF-1)-dependent binding of aminoacyl-tRNA, and EF-2-catalyzed GTP hydrolysis and translocation (Wool *et al.*, 1992). This specific action of ribotoxins is so effective that a single molecule of α-sarcin is enough to kill a cell (Lamy *et al.*, 1992). It was this unique potency and specificity against ribosomes that prompted us to designate them as 'natural killers'.

Ribosome-inactivating proteins (RIPs) are a group of highly specialized toxic proteins (Stirpe *et al.*, 1988, 1992; Nielsen & Boston, 2001) produced by plants and fungi that inactivate ribosomes by acting on the same unique rRNA gene structure as ribotoxins do (Schindler & Davies, 1977; Endo & Wool, 1982; Endo *et al.*, 1987; Correll *et al.*, 1998, 1999; Mears *et al.*, 2002). Ribotoxins would be also RIPs from this point of view, but some authors claim (Nielsen & Boston, 2001; Peumans *et al.*, 2001) that the name RIP should be restricted to plant *N*-glycosidases, best represented by ricin, that depurinate a single nucleotide contiguous to the phosphodiester bond cleaved by ribotoxins (Endo *et al.*, 1987; Endo & Tsurugi, 1987). These are the reasons why the conserved rRNA gene sequence targeted by RIPs and ribotoxins has come to be universally known as the sarcin–ricin loop, or SRL (Fig. 2).

Many prokaryotes encode both a labile antitoxin and a stable toxin under the control of a single operon. These toxin–antitoxin (TA) systems constitute another interesting family of toxic endo-RNases (Christensen *et al.*, 2003; Muñoz-Gómez *et al.*, 2005; Condon, 2006; Kamphuis *et al.*, 2006; Luna-Chávez *et al.*, 2006). The antitoxin is synthesized in equimolecular amounts to the toxin, thus inhibiting its ribonucleolytic action. Once the cells are under a variety of stress conditions, the antitoxin is inactivated and the action of the toxin promotes cellular growth arrest in preparation for a more favorable situation. The target of these proteins is not completely established, but mRNA seems to be the best candidate according to the data so far published. It cannot be ruled out, however, that the ribosome, or most probably the translation complex, exerts some type of stimulating or modulating activity (Christensen & Gerdes, 2003). Thus, these endo-RNases can also be considered as modulators of protein biosynthesis and, in this regard, they have a functional connection with fungal ribotoxins. Actually, they share cleavage mechanisms, as they also behave as cyclizing

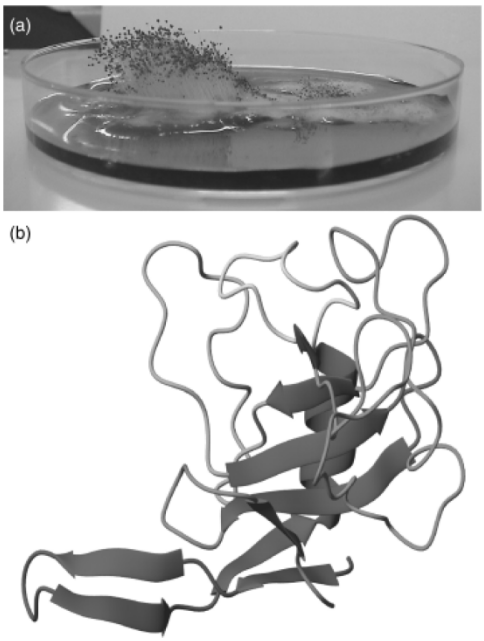

Fig. 1. (a) Photograph of a culture of *Aspergillus giganteus* MDH 18894. (b) Three-dimensional structure of α-sarcin (PDB ID 1DE3): The diagram was generated with the molmol program (Koradi *et al.*, 1996).

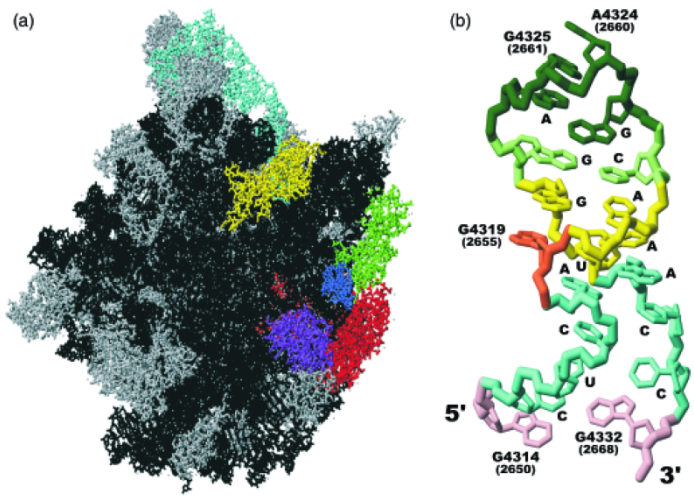

Fig. 2. (a) Diagram showing the structure of the *Halobacterium marismortui* ribosome large subunit (PDB ID 1JJ2): black, 23S RNA gene; cyan, 5S RNA gene; blue, SRL; red, ribosomal protein L3; green, ribosomal protein L6; yellow, ribosomal protein 10e; purple, ribosomal protein L14; gray, other ribosomal proteins. (b) Diagram showing the structure of the SRL (Correll *et al.*, 1998). Numbers correspond to rat or *Escherichia coli* (in brackets) nucleotide positions within the 28S (23S) rRNA gene. The bond cleaved by ribotoxins is that on the 3′-side of G4325 (2661) (dark green). Ricin depurinates A4324 (2660) (dark green). The bulged G is G4319 (2655) (orange). The Watson–Crick region of the hairpin (violet), the flexible region (cyan), the G-bulged cross-strand stack (yellow) and the tetraloop (green) are colored. The diagrams were generated with the molmol program (Koradi *et al.*, 1996).

RNases, as ribotoxins do (see below). However, the similarities end there, as known ribotoxins are not produced by prokaryotes, and use a different pair of side chain residues for the acid–base catalysis, and no specific antitoxin production has been so far reported (Martínez-Ruiz *et al.*, 1998, 2001; Kamphuis *et al.*, 2006).

In addition to their ribonucleolytic activity, ribotoxins cross lipid membranes in the absence of any known protein receptor (Oñaderra *et al.*, 1993; Gasset *et al.*, 1994; Martínez-Ruiz *et al.*, 2001). Thus, although any ribosome could be potentially inactivated by these proteins, owing to the universal conservativeness of the SRL, they are especially active on transformed or virus-infected cells (Olson *et al.*, 1965; Fernández-Puentes & Carrasco, 1980; Olmo *et al.*, 2001). This observation has been explained in terms of altered permeability of these cells combined with the ability of ribotoxins to interact with acid phospholipid-containing membranes (Gasset *et al.*, 1989, 1990; Martínez-Ruiz *et al.*, 2001; Olmo *et al.*, 2001).

α-Sarcin, restrictocin and Aspf1 are the most exhaustively characterized ribotoxins (Arruda *et al.*, 1992; Gasset *et al.*, 1994; Wool, 1997; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001; García-Ortega *et al.*, 2005), but many others have been identified and partially characterized in different fungal species (Lin *et al.*, 1995; Parente *et al.*, 1996; Huang *et al.*, 1997; Wirth *et al.*, 1997; Martínez-Ruiz *et al.*, 1999a, b). These studies have suggested a high degree of conservation among ribotoxins, as those characterized display amino acid sequence similarities above 85% (Fig. 3). However, hirsutellin A (HtA), an extracellular ribonucleolytic protein produced by the invertebrate fungal pathogen *Hirsutella thompsonii*, has been recently demonstrated to be a ribotoxin (Herrero-Galán *et al.*, 2007), and it displays only about 25% sequence identity with previously known family members (Boucias *et al.*, 1998; Martínez-Ruiz *et al.*, 1999a; Herrero-Galán *et al.*, 2007). This suggests that the presence of ribotoxins among fungi is more widespread than initially considered (Martínez-Ruiz *et al.*, 1999b). A specific RNase purified from mature seeds of oriental arbovitae (*Biota orientalis*) has also been reported to cleave a single phosphodiester bond of 28S rRNA gene in rat ribosomes but in a different region from the SRL, although spatially close to it (Xu *et al.*, 2004).

Aspergilli are a ubiquitous and complex group of filamentous fungi containing more than 185 species, including 20 human pathogens as well as others used for the industrial production of foods and enzymes. The publication of the genome sequence of the model organism *Aspergillus nidulans* (Galagan *et al.*, 2005) has created huge expectations regarding advances in our knowledge of the biology of these microorganisms. A comparative genomic study involving two other species, *Aspergillus fumigatus* and *Aspergillus oryzae* (Machida *et al.*, 2005; Nierman *et al.*, 2005), has also been reported. *Aspergillus nidulans* does not produce any ribotoxin, whereas *A. fumigatus*, a serious human pathogen, produces Aspf1, one of the best known ribotoxins (Moser *et al.*, 1992). *Aspergillus oryzae* (Machida *et al.*, 2005) is used in the production of sake, miso and soy sauce, and also of

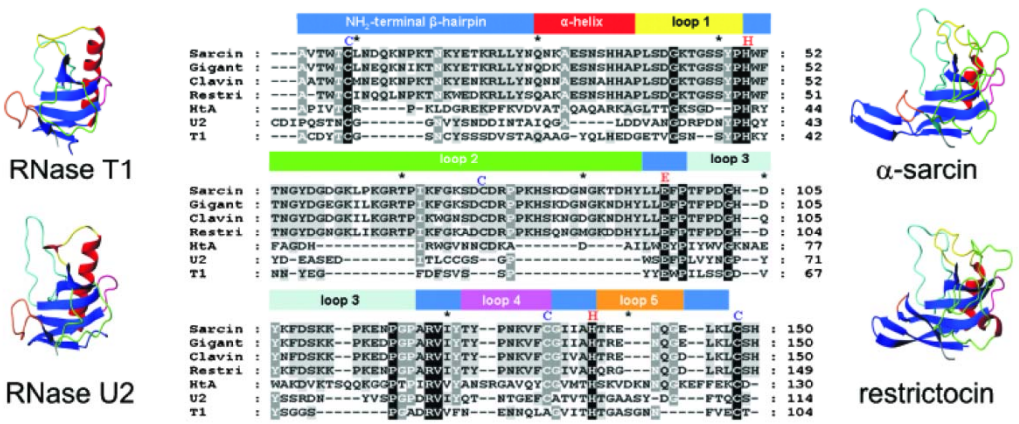

Fig. 3. (a) Sequence alignment of several different ribotoxins (α-sarcin, gigantin, clavin, restrictocin, and HtA) and RNases T1 and U2. Cysteine residues of ribotoxins (blue) and the three proved, or presumed, catalytic residues of all the proteins (in red) are indicated. Diagrams showing the three-dimensional structures of α-sarcin, restrictocin and RNases T1 and U2 are also shown. Color codes are as in Fig. 1. Three-dimensional structures of proteins were fitted to the atomic coordinates of the active site residues (α-sarcin: 48, 50, 96, 121, 137, 145; restrictocin: 47, 49, 95, 120, 136, 144; RNase T1: 38, 40, 66, 77, 92, 100; RNase U2: 39, 41, 62, 85, 101, 110) and common disulfide bridges of the four proteins (α-sarcin, 6–148; restrictocin, 5–147; RNase T1, 6–103; RNase U2, 9–113) (root mean square deviation of the fitting, 1.877). The diagrams and fittings were generated with the molmol program (Koradi *et al.*, 1996).

RNase T1 (Sato & Egami, 1957), one of the most exhaustively characterized proteins. RNase T1 is indeed the best known member of the family of fungal extracellular RNases (Yoshida, 2001; Loverix & Steyaert, 2001), a group that obviously includes ribotoxins. All of them show a high degree of sequence (Sato & Uchida, 1975; Sacco *et al.*, 1983; Martínez-Ruiz *et al.*, 1999a, b) and structural similarity (Pace *et al.*, 1991; Noguchi *et al.*, 1995; Yang & Moffat, 1996; Campos-Olivas *et al.*, 1996a, b; Pérez-Cañadillas *et al.*, 2000) (Fig. 3), but, apart from ribotoxins, none of these RNases has been reported to show cytotoxic activity.

Besides RNase T1, RNase U2 from *Ustilago sphaerogena* (Fig. 3) (Arima *et al.*, 1968a, b; Sato & Uchida, 1975) also stands out as the nontoxic microbial extracellular RNase most closely related to ribotoxins from a phylogenetic point of view (Sacco *et al.*, 1983; Martínez del Pozo *et al.*, 1988; Martínez-Ruiz *et al.*, 1999a, b, 2001). RNase U2 is a small and highly acidic protein that shows a strong preference for 3′-linked purine nucleotide phosphodiester linkages (Rushizky *et al.*, 1970; Uchida *et al.*, 1970), which is rather unusual within the group of microbial RNases. RNase T1, for example, shows strict specificity for the guanyl group. Both enzymes also differ in their optimum pH values, which are acid for RNase U2 and neutral for RNase T1, but both are cyclizing enzymes, cleaving RNA in two separate steps, transphosphorylation and hydrolysis (Yasuda & Inoue, 1982).

Ribotoxins are larger proteins, generally basic, that contain longer and charged loops that are not present in the noncytotoxic fungal RNases (Fig. 3), suggesting that these loops are the structural basis of their toxicity (Martínez del Pozo *et al.*, 1988). It would appear that an RNase T1-like RNase had acquired ribosome specificity by the insertion of short recognition domains that would target it to cleave more specific substrates. Thus, the study of the evolution and mechanism of action of ribotoxins is of particular interest, as they appear to be naturally engineered targeted toxins evolved from the other microbial nontoxic RNases to enter cells and specifically inactivate the ribosomes (Lamy *et al.*, 1992; Kao & Davies, 1995). Identification of the structural features that have allowed these proteins to become such efficient natural killers would be a major step towards their utilization, native or modified, as weapons against different human pathologies.

Structural features

Ribotoxins are synthesized as precursors that mature into cellular membrane compartments (Endo *et al.*, 1993a, b). Consequently, there has been speculation for a long time about the mechanism developed by the producing fungi in order to overcome the toxicity of these proteins, as their own ribosomes are also sensitive to the action of the toxins (Miller & Bodley, 1988). There is no evidence for the simultaneous production of any antitoxin or protein inhibitor that could block their cytotoxic action before they are secreted to the extracellular medium (Martínez-Ruiz *et al.*, 1998b), as happens with some bacterial ribonucleolytic toxins (Muñoz-Gómez *et al.*, 2005; Condon, 2006; Kamphuis *et al.*, 2006; Luna-Chávez *et al.*, 2006). In addition, characterization of pro-α-sarcin, produced in *Pichia pastoris*, revealed that it is ribonucleolytically active (Martínez-Ruiz *et al.*, 1998). Thus, the data so far accumulated suggest that protection of the producing cells against the toxic effects of ribotoxins must rely on efficient recognition of their signal sequences, followed by adequate compartmentalization before they are secreted to the extracellular medium.

The complete or partial amino acid sequences of several ribotoxins have been determined (Rodríguez *et al.*, 1982; Sacco *et al.*, 1983; López-Otín *et al.*, 1984; Fernández-Luna *et al.*, 1985; Arruda *et al.*, 1990; Wirth *et al.*, 1997; Martínez-Ruiz *et al.*, 1999a, b). They show a high degree of identity in their *c.* 150 amino acid sequence (Fig. 3), including the conservation of their two disulfide bridges (Martínez del Pozo *et al.*, 1988; Martínez-Ruiz *et al.*, 2001). This observation includes HtA (Martínez-Ruiz *et al.*, 1999a), although it is 20 residues shorter than the other known ribotoxins. Sequence differences are mainly concentrated at the loops of the ribotoxins (Martínez-Ruiz *et al.*, 1999a) (Fig. 3).

This similarity is also manifested in the three-dimensional structure of the two ribotoxins studied at this level, restrictocin (Yang & Moffat, 1996; Yang *et al.*, 2001) and α-sarcin (Pérez-Cañadillas *et al.*, 2000, 2002; García-Mayoral *et al.*, 2005a, b). For α-sarcin, nuclear magnetic resonance (NMR) and other techniques have been used to make a very detailed map of its structural and dynamic properties (Campos-Olivas *et al.*, 1996a, b; Pérez-Cañadillas *et al.*, 2000, 2002; García-Mayoral *et al.*, 2005a, b). The elucidation of its three-dimensional structure (Fig. 1) revealed that it folds into an α+β structure with a central five-stranded antiparallel β-sheet and an α-helix of almost three turns. The sheet is composed of the strands β3, β4, β5, β6 and β7 arranged in a $-1, -1, -1, -1$ topology (Figs 1 and 3) (Campos-Olivas *et al.*, 1996a, b; Pérez-Cañadillas *et al.*, 2000). It is highly twisted in a right-handed sense, defining a convex face against which the α-helix is orthogonally packed, and a concave surface that holds the active site residues His50, Glu96, Arg121 and His137, with their side chains projecting outwards from the cleft (Fig. 4). In addition, residues 1–26 form a long β-hairpin that can be considered as two consecutive minor β-hairpins connected by a hinge region. The first one is closer to the open end of the hairpin, whereas the second sub-β-hairpin is formed by two short strands β1b and β2b connected by a type I β-turn. This last part of the N-terminal hairpin juts out as a solvent-

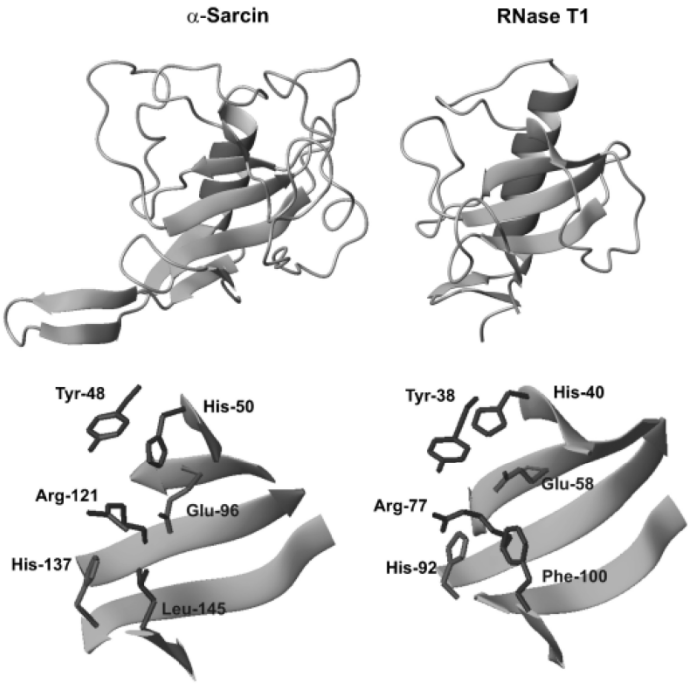

Fig. 4. Representation of the geometric arrangement of the side chain residues found in the active sites of α-sarcin and RNase T1. Only the side chains of the catalytic residues directly involved in the mechanism of general acid–base cleavage are shown. The three-dimensional structures of both proteins are also shown. The diagrams were generated with the molmol program (Koradi *et al.*, 1996).

exposed protuberance, a detail that it is important for its function, as explained below. α-Sarcin and restrictocin show almost identical structures (Fig. 3), but some small differences are observed concerning their long nonstructured loops and especially the above-mentioned N-terminal β-hairpin, a region of high mobility (Pérez-Cañadillas *et al.*, 2002), which is lacking in the restrictocin crystalline structure (Yang & Moffat, 1996). The remaining stretches of its sequence form large loops connecting the secondary structure elements (Fig. 1). Despite the exposed character of these loops and their lack of repetitive secondary structure, their conformations are well defined. They are maintained by networks of intraloop and interloop interactions, including hydrogen bonds, hydrophobic interactions, and salt bridges (Yang & Moffat, 1996; Pérez-Cañadillas *et al.*, 2000). From a dynamic point of view, NMR measurements have shown that α-sarcin behaves as an axial symmetric rotor of the prolate type, and that it is composed of a rigid hydrophobic core and some exposed segments, mostly the loops, which undergo fast (picosecond to nanosecond) internal motions (Pérez-Cañadillas *et al.*, 2002).

Ribotoxins share this structural core with nontoxic RNases of the RNase T1 family, in good agreement with the observed sequence similarity (Figs 3 and 4). For example, comparison of the three-dimensional structures of α-sarcin and restrictocin with those of RNase T1 and RNase U2 reveals that the four proteins share identical regular secondary structure elements despite their different amino acid sequence lengths, including the geometric arrangement of the residues involved in the active site (Figs 3 and 4). Thus, all fungal extracellular RNases whose three-dimensional structure is known exhibit quite different enzymatic specificities, but all of them share this common structural fold concerning the architecture and connectivity of the secondary structure elements (Yang & Moffat, 1996; Campos-Olivas *et al.*, 1996b; Pérez-Cañadillas *et al.*, 2000; Martínez-Ruiz *et al.*, 2001). The most significant structural differences among them are, again, related to both the presence of a longer N-terminal β-hairpin in ribotoxins and the different length and charge of their unstructured loops (Figs 3 and 4).

Loop 2 of α-sarcin (Thr53 to Tyr93) (Fig. 3) deserves especial emphasis, because it exhibits a well-defined conformation with functional implications (Pérez-Cañadillas *et al.*, 2000). It is highly mobile, rich in Gly and positively charged residues, and largely solvent exposed. In this loop, the stretch comprising residues 52–54 is essentially frozen within the molecular framework (Pérez-Cañadillas *et al.*, 2002), Asn54 being a conserved residue among fungal extracellular RNases (Mancheño *et al.*, 1995a) that establishes a hydrogen bond between its amide side chain proton and the carbonyl group of Ile69, these protons being very resistant to exchange with the solvent. This interaction is,

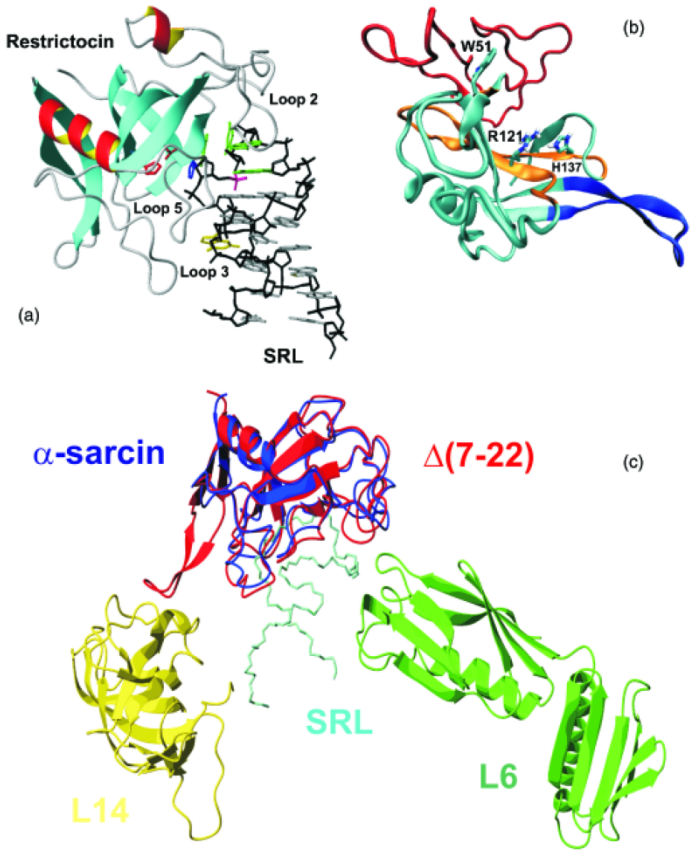

Fig. 5. (a) Diagram showing the crystalline structure of an SRL analog–restrictocin complex (Yang *et al.*, 2001) (PDB accession number 1JBS). The analog structure is distorted in comparison to the wild-type SRL, and this explains the absence of cleavage, which allowed crystallization of the complex. The side chains of His49 (blue), Glu95 (red), and His136 (red) are also shown. The RNA backbone is shown is black, with the bases in gray, except for the bulged G in yellow, the tetraloop in green, and the phosphate group of the bond susceptible to cleavage in magenta. The loops equivalent to α-sarcin's loops 2, 3 and 5 are also indicated. (b) Diagram showing the α-sarcin regions presumably involved in the establishment of interactions with phospholipid bilayers: purple, residues 7–22; orange, residues 116–139 and 51; red, residues 53–93 (loop 2). The side chains of Trp51, Arg121 and His137 are also shown. (c) Minimized docking model showing the interaction of wild-type (PDB ID 1DE3) and Δ(7–22) α-sarcin (PDB ID 1R4Y) with the SRL (PDB ID 430D) and the *Halobacterium marismortui* ribosomal proteins L6 and L14 (García-Mayoral *et al.*, 2005a, b). Diagrams were generated with molmol (a, c) (Koradi *et al.*, 1996) and vmd (b) (Humphrey *et al.*, 1996) programs.

indeed, conserved in the other RNases of the RNase T1 family (Sevcik *et al.*, 1991; Pfeiffer *et al.*, 1997, Hebert *et al.*, 1998). Docking studies have also suggested that the segment formed by residues 51–55 of α-sarcin (Fig. 3) could specifically interact with the SRL in the vicinity of the scissile bond (Pérez-Cañadillas *et al.*, 2000), a prediction that was later confirmed by X-ray crystallography (Fig. 5a) (Yang *et al.*, 2001). In relation to this, three Lys residues of loop 3 (Lys111, Lys112 and Lys114) seem to be of especial importance, as they contact the identity element of the ribosomal SRL region, the bulged-G in the S-turn (see below and Fig. 5a) (Yang & Moffat, 1996; Pérez-Cañadillas *et al.*, 2000; Yang *et al.*, 2001).

It is also remarkable that an N-terminal deletion mutant Δ(7–22) of α-sarcin (García-Ortega *et al.*, 2002) retained the same conformation as the wild-type protein, as ascertained from its spectroscopic characterization (García-Ortega *et al.*, 2002) and three-dimensional structure in solution (García-Mayoral *et al.*, 2004). Docking and enzymatic studies have revealed that this N-terminal β-hairpin (positions 7–22) of α-sarcin could establish interactions with ribosomal proteins in order to direct the ribotoxin to the SRL region of the ribosome (García-Ortega *et al.*, 2002; García-Mayoral *et al.*, 2005b) (Fig. 5).

α-Sarcin is a highly charged protein, with a high isoelectric point. The high content of positively charged residues is probably required for recognizing and binding to not only its highly negatively charged target, the rRNA gene, but also the cellular membranes. It contains eight Tyr and two Trp residues, which have been spectroscopically explored. By using UV absorbance, fluorescence emission and circular dichroism (CD) measurements, five different pH-induced conformational transitions, corresponding to p*Ka* values of 2.5, 4.5, 8.0, 10.2, and 11.4, were initially described (Martínez del Pozo *et al.*, 1988). The two latter ones (10.2 and 11.4) corresponded to two different Tyr populations of different solvent accessibility. The transition at p*Ka* 8.0 was assigned to the α-amino group of the N-

terminal residue, Asp and Glu residues deprotonated at pH 4.5, and p*K*a values of 2.5 and 10.2 were considered to be denaturing transitions (Martínez del Pozo *et al.*, 1988). This characterization later became much more detailed, when the p*K*a values of all aspartic acid, glutamic acid and histidine residues of α-sarcin were determined by NMR; it was found that many of them, including several at the active site, are highly perturbed (Pérez-Cañadillas *et al.*, 1998). Much more recently, the p*K*a values of all titratable residues have also been systematically measured, or predicted when direct measurement was not possible because of the unfolding of the protein (García-Mayoral *et al.*, 2003). These measurements and predictions were also extended to a series of active site variants (E96Q, H50Q, H137Q, and H50/137Q) (García-Mayoral *et al.*, 2003, 2006). This detailed characterization at the level of individual residues was completed by determining the tautomeric state of all the side chain histidine residues (Pérez-Cañadillas *et al.*, 2003).

α-Sarcin's two Trp residues, at positions 4 and 51, are conserved in all ribotoxins known so far (Fig. 3). Characterization of mutants where one or both of these two residues were substituted by Phe (single mutants W4F and W51F, and the double mutant W4/51F) showed that they are not required for the highly specific ribonucleolytic activity of the protein, although the mutant forms involving Trp51 showed decreased activity (De Antonio *et al.*, 2000). More importantly, it was shown that Trp51 is responsible for most of the near-UV CD of the protein, and also contributes to the overall ellipticity of the protein in the peptide bond region, but does not show fluorescence emission (De Antonio *et al.*, 2000).

Finally, it is also important, from a structural point of view, to mention that the active site of α-sarcin is composed of at least residues Tyr48, His50, Glu96, His137, Arg121, and Leu145, although only three of them (His50, Glu96, and His137) are directly involved in proton transfer steps in the catalytic mechanism (Lacadena *et al.*, 1999; Martínez-Ruiz *et al.*, 2001). As mentioned above, they are located in the central β-sheet, and their side chains point towards the concave face of the protein structure (Fig. 4). The most representative characteristics of this active site would be: (1) high density of charged residues; (2) unusual p*K*a values of His50, Glu96, and His137; (3) unusual Nδ tautomeric forms adopted by His50 and His137, a common feature of microbial RNases; (4) the presence of a structurally important hydrogen bond between the catalytic His137 and a backbone oxygen in loop 5; and (5) low surface accessibility of all titratable atoms (Pérez-Cañadillas *et al.*, 1998, 2000, 2003).

Crossing membranes

The antitumor activity of α-sarcin can be explained by its unique ribonucleolytic activity after its selective passage across some cell membranes. Thus, although the SRL is a universally conserved structure, cells are only killed if ribotoxins cross their membranes to gain access to the ribosomes. As no protein receptors have so far been reported for α-sarcin, the toxic specificity must be related to a differential interaction with the lipid components of the membranes. Long ago, it was shown that α-sarcin was a powerful inhibitor of protein synthesis in picornavirus-infected cells (Fernández-Puentes & Carrasco, 1980), and that ionophores (Alonso & Carrasco, 1981, 1982), external ATP (Otero & Carrasco, 1986) or phospholipase C treatment (Otero & Carrasco, 1988) targeted mammalian cells to α-sarcin entry. All these observations were interpreted in terms of the existence of altered membrane permeability.

The use of lipid model systems proved that α-sarcin interacts specifically with negatively charged phospholipid vesicles at neutral or slightly acidic pH, resulting in protein–lipid complexes that can be isolated by centrifugation in a sucrose gradient (Gasset *et al.*, 1989). Binding experiments revealed a strong ribotoxin–lipid vesicle interaction ($K_d = 60.0$ nM) that caused vesicle aggregation, followed by their fusion into much larger lipidic structures (Gasset *et al.*, 1989). The minimum phosphatidylcholine/phosphatidylglycerol molar ratio required for this behavior was 1 : 10, and it was dependent on neither the length nor the degree of unsaturation of the phospholipid acyl chain, being more effective at temperatures above the melting temperature of the phospholipid used. Saturation was reached at phospholipid/protein molar ratios of 50 : 1, and the effect was maximal at 0.15 M ionic strength. It was, however, abolished at basic pH (Gasset *et al.*, 1990).

Light-scattering stopped-flow kinetic studies of the α-sarcin–vesicle interaction revealed that the initial step was the formation of a vesicle dimer maintained by protein–-protein bridges (Mancheño *et al.*, 1994). Once the aggregation had started, lipid mixing occurred between the bilayers of aggregated vesicles, as would be expected with fusing liposomes. In fact, this fusion was triggered by the destabilizing effect of the protein, which simultaneously suffered conformational changes upon binding to the vesicles (Mancheño *et al.*, 1994), as revealed by CD, fluorescence emission, and attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) (Gasset *et al.*, 1991b). These conformational changes suggested an increase in the α-helix content that, together with the other spectroscopic changes observed, was interpreted in terms of shielding from polar groups caused by the lipids, which would promote intrachain hydrogen bonding and decreased static quenching (Gasset *et al.*, 1991b). Indeed, the peptide bonds of the protein were protected against trypsin hydrolysis upon binding to these vesicles (Gasset *et al.*, 1989; Oñaderra *et al.*, 1989), despite the high number of basic residues present along its sequence (Sacco *et al.*, 1983). Freeze-fracture electron micrographs corroborated this fusogenic

effect, as at the highest phospholipid/protein molar ratio employed (50 : 1), there was a complete absence of small vesicular structures; instead, lipids were exclusively organized in planar sheets, indicating that the fusion processes had gone to completion (Gasset *et al.*, 1990). As a final step, and most probably as a consequence of the formation of these unstable large structures, α-sarcin also modified the permeability of the membranes, causing the leakage of calcein in dye-trapped phosphatidylglycerol vesicles (Gasset *et al.*, 1990). Fluorescence depolarization measurements, differential scanning calorimetry and labeling with photoactive phospholipids revealed that α-sarcin, a water-soluble and hydrophilic protein, interacts with phospholipid bilayers through a combination of electrostatic and hydrophobic forces (Gasset *et al.*, 1991a). According to this hypothesis, the protein would then initially be adsorbed to the charged polar head groups of the phospholipids, and then would partially penetrate the interface of the bilayer to interact with a portion of the lipid hydrocarbon chains (Gasset *et al.*, 1991a). All these observations were consistent with an intercalation of the protein into the lipid matrix, promoting fusion and permeability changes in the bilayers, processes that would presumably be involved in the passage of the protein across the membranes of its target cells. A higher content of negatively charged phospholipids, such as phosphatidylserine, has been reported in transformed cell membranes (Connor *et al.*, 1989; Gasset *et al.*, 1989, 1990; Zachowski, 1993). Unfortunately, there is no direct evidence yet that this abundance of acidic phospholipids is the main explanation for the antitumor activity of α-sarcin.

In good agreement with this hypothesis, the innate ability of α-sarcin to translocate across a phospholipid membrane, if it is acidic enough, in the absence of any other protein was also demonstrated, using two different types of assay (Oñaderra *et al.*, 1993). First, the protein was completely degraded when added externally to asolectin vesicles containing encapsulated trypsin, an experiment that was performed in the presence of such an external excess of trypsin inhibitor that degradation by traces of leaked protease was not possible. Second, externally added α-sarcin was also capable of cleaving encapsulated baker's yeast tRNA molecules in a protein concentration-dependent manner (Oñaderra *et al.*, 1993).

With regard to the protein regions involved in the interaction, the first hints were obtained using water-soluble synthetic peptides corresponding to sequences within the main β-sheet of α-sarcin. Some of these peptides, one of them only nine residues long, were shown to be able to mimic, at least qualitatively, the effects produced by the complete protein on acid phospholipid vesicles, indicating that this region of the protein (residues 116–139) is probably involved in its interaction with the cell membranes (Fig. 5b) (Mancheño *et al.*, 1995b, 1998a). These conclusions were indeed compatible with the observation that a denatured form of α-sarcin, containing β-strands as the only regular secondary structure elements, promoted destabilization of the hydrophobic core of bilayers (Gasset *et al.*, 1995). Using the Trp mutants mentioned above, it was also shown that neither Trp4 nor Trp51 were required for the interaction of α-sarcin with lipid membranes (aggregation and fusion of vesicles) (De Antonio *et al.*, 2000). However, this interaction promoted a large increase in the quantum yield of Trp51, the residue located in the β-sheet of the protein (Fig. 5b), and its fluorescence emission was simultaneously quenched by anthracene incorporated into the hydrophobic region of such bilayers. Furthermore, a study of mutants affecting α-sarcin active site residue Arg121 (R121 K and R121Q), also located at the major β-sheet (Figs 4 and 5b), showed that the loss of the positive charge at that position produced a dramatic impairment of the protein's ability to interact with phospholipid membranes (Masip *et al.*, 2001). This interesting result led to the proposal that proteins that had evolved to interact with RNA, such as ribotoxins, would have developed structural and chemical determinants to recognize polyphosphate lattices that might, indeed, allow the recognition of a phospholipid bilayer (Masip *et al.*, 2001). Interestingly, when the crystalline structure of restrictocin was solved, the equivalent residue (Arg120) was found to be hydrogen bonded to a cocrystallized phosphate molecule at its active site (Yang & Moffat, 1996). In summary, these results indicated that this β-sheet, predicted to be one of the scarcest apolar regions of the protein (Martínez del Pozo *et al.*, 1988; Mancheño *et al.*, 1995b), was in fact located within the hydrophobic core of the bilayer following protein–vesicle interaction (Fig. 5b) (De Antonio *et al.*, 2000).

Other than this hydrophobic core, mutations affecting single residues located at the N-terminal β-hairpin of α-sarcin (Lys11 and Thr20) and the deleted Δ(7–22) variant suggested that this protein portion would be another region involved in the interaction with cell membranes (García-Ortega *et al.*, 2001, 2002), as they displayed a different pattern of interaction with the lipid vesicles (Fig. 5b). When restrictocin was the protein assayed, it also behaved differently from wild-type α-sarcin (García-Ortega *et al.*, 2001). It is noteworthy that α-sarcin and restrictocin sequences differ in only 20 residues, and six of these changes are concentrated at the N-terminal β-hairpin (Fig. 3). In agreement with this idea, the Δ(7–22) α-sarcin showed behavior compatible with the absence of one vesicle-interacting protein region (García-Ortega *et al.*, 2002).

Finally, loop 2 has been proposed by several authors (Yang & Moffat, 1996; Martínez del Pozo *et al.*, 1988; Kao & Davies, 1999; Pérez-Cañadillas *et al.*, 2000) to also be one of the protein regions involved in the interaction with lipids

(Fig. 5b). The differences between the NMR-refined structure of this loop in α-sarcin and restrictocin (García-Mayoral *et al.*, 2005a, b) could help to explain their distinct behavior when translocating across cell membranes, although this possibility has not been directly studied yet.

Enzymatic properties

The enzymatic activity of ribotoxins remained obscure for a long time after their discovery (Lamy *et al.*, 1992). Then, in 1977, Schindler and Davies published the observation that α-sarcin was able to inactivate both *Saccharomyces cerevisiae* and *Escherichia coli* ribosomes, although with different efficiencies (Schindler & Davies, 1977). Surprisingly, neither intact yeast or bacteria nor HeLa cells were susceptible to the toxicity exerted by α-sarcin, suggesting that they were refractory to the entrance of the protein. A more detailed study concluded that α-sarcin's inactivation of purified ribosomes affected EF-2-catalyzed GTP hydrolysis and translocation. Finally, separation of the rRNA gene species by gel electrophoresis after incubation of yeast 80S ribosomes with the toxin resulted in the appearance of an extra fragment about 300 nucleotides long (the so-called α-fragment), corresponding to the 3′-end of the 28S rRNA gene (Fig. 6a) (Schindler & Davies, 1977). Further experiments showed that α-sarcin cleaved the phosphodiester backbone at the 3′-side of G2661 (*E. coli* numbering) (Endo & Wool, 1982), whereas ricin depurinated the N-glycosidic linkage between the ribose sugar and the base moieties corresponding to the 5′-adjacent A2660 (both positions corresponding to G4325 and A4324 in 28S rRNA gene) (Fig. 2) (Endo & Tsurugi, 1987; Endo *et al.*, 1987).

Therefore, ribotoxins are highly specific RNases against cell-free intact ribosomes, and they retain this specificity when assayed against naked rRNA gene containing the SRL. However, they can also cause extensive progressive digestion of total or 28S rRNA with no formation of the α-fragment, when used at higher concentrations (Endo *et al.*, 1993a, b; Wool, 1996, 1997). Even DNA has been shown to be digested by α-sarcin when large enzyme/substrate ratios are assayed (Wool, 1984; Endo *et al.*, 1993a, b). This nonspecific activity has been taken advantage of in the employment of some other, much less specific, ribonucleolytic assays, apart from those based on following the release of the α-fragment. The lack of biological significance, due to the loss of specificity, and the much higher than catalytic concentrations needed, are compensated for by much easier quantitation of the results, as well as the possibility of analyzing the products, or even intermediates, of the reaction. Thus, although they are less specific, these assays have contributed significantly to the detailed study of the cleavage mechanism of ribotoxins (Lacadena *et al.*, 1994, 1998; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001).

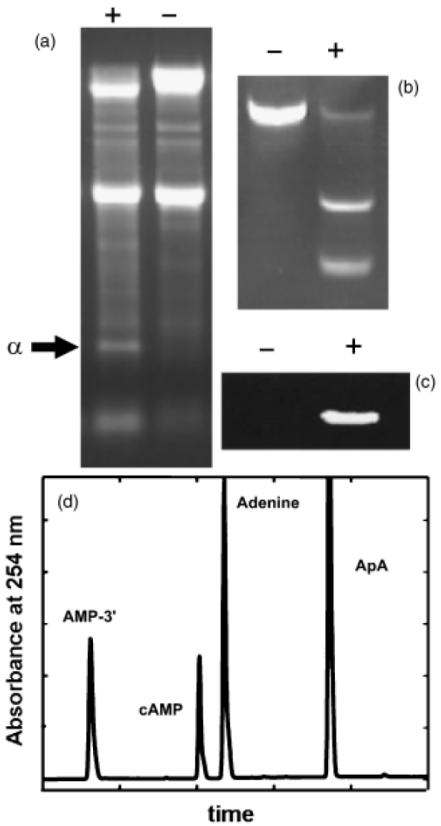

Fig. 6. Examples of the different enzymatic assays used to study ribotoxin ribonucleolytic activity. The presence (+) or absence (−) of α-sarcin in the assay is indicated. (a) Specific cleavage of rabbit ribosomes. The α-fragment is indicated by an arrow. (b) Specific cleavage of a 35-mer SRL-like oligonucleotide. (c) Zymogram against poly(A). (d) HPLC resolution of the products produced after incubation of α-sarcin with ApA.

Four different types of enzymatic assay are usually performed (Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). The first, and most specific, is one that uses natural substrates, purified ribosomes or, at least, a cell-free reticulocyte lysate (Kao *et al.*, 2001). The highly specific action can be then visualized by detecting the release of a 300–400-nucleotide (depending on the ribosome source) α-fragment on a denaturing agarose gel stained with ethidium bromide (Fig. 6a). The sensitivity of this assay has recently been improved by the detection of this α-fragment by hybridization with a specific ^{32}P-radiolabeled DNA probe (Korennykh *et al.*, 2006).

In decreasing order of complexity, and therefore of specificity, the second assay frequently used is based on the

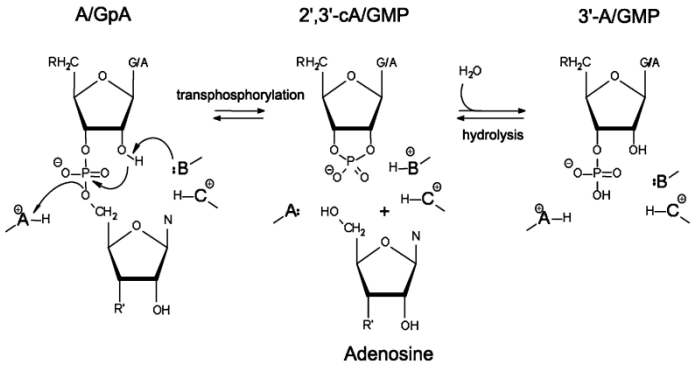

Fig. 7. Proposed mechanism for the catalytic mechanism of cyclizing RNases against a dinucleotide substrate (ApA or GpA). A transphosphorylation process (in which the corresponding 2′,3′ cyclic mononucleotide and adenosine are produced) is followed by hydrolysis of the cyclic nucleotide to produce the corresponding 3′-mononucleotide. (A), (B) and (C) are His92, Glu58 and His40 in RNase T1 (Steyaert, 1997), and His137, Glu96 and His50 in α-sarcin (Lacadena *et al.*, 1999), respectively.

employment of short oligoribonucleotides mimicking the SRL sequence and structure (SRL-like oligos). Ribotoxins cleave these SRL-like oligos specifically, producing only two smaller fragments, which can be fractionated on a polyacrylamide gel (Fig. 6b) (Endo *et al.*, 1988; Wool *et al.*, 1992), although this cleavage is several orders of magnitude less efficient than that produced on intact ribosomes (Glück & Wool, 1996; Wool, 1997).

The third, and much less specific, assay is a zymogram (Fig. 6c), in which the ribonucleolytic activity is shown against a homopolymer, such as poly(A) or poly(I), embedded in a polyacrylamide gel after electrophoretic separation of the proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and convenient refolding by elimination of the detergent. In some instances, this type of assay can be also performed in solution, using ultrafiltration devices to fractionate the small oligonucleotides produced by the ribonucleolytic reaction (Kao *et al.*, 2001). The advantage of the zymogram is its additional use as a homogeneity control of the protein sample.

The fourth assay is based on the fact that ribotoxins are also capable of hydrolyzing different dinucleoside (or dinucleotide) phosphates, such as ApA (or ApAp), although with very low efficiency (Lacadena *et al.*, 1998). This type of substrate should be considered as just containing the minimal and essential elements needed to be cleavable by a RNase. The advantage in this case is that the products, substrates and intermediates of the reaction can be separated and quantitated by HPLC (Fig. 6d), providing information about the different steps (Lacadena *et al.*, 1998).

A combination of all these different activity assays, and the production and characterization of many site-directed and randomly produced mutants (Yang & Kenealy, 1992a, b; Lacadena *et al.*, 1995, 1999; Kao *et al.*, 1998), have allowed the determination of not only the ribotoxin residues involved in the catalytic reaction, but also their different roles during the cleavage of a phosphodiester bond. The non-cytotoxic microbial RNases T1 and U2 have been of great help as reference models. The enzymatic mechanism of RNase T1, for example, has been clearly established (Fig. 7), as have the roles of most of the residues forming its active site (Steyaert, 1997; Loverix & Steyaert, 2001; Yoshida, 2001). Accordingly, this enzyme performs the general acid–-base type endonucleolytic cleavage of RNA in two steps. First, there is a transphosphorylation reaction to form a 2′,3′-cyclic phosphate intermediate. Second, this intermediate is hydrolyzed to the corresponding 3′-phosphate (Fig. 7). The appearance of this cyclic intermediate, common to all RNases of the RNase T1 family so far studied, including RNase U2, is implicit in the denomination of all these enzymes as cyclizing RNases. Analysis of the cleavage reactions performed by α-sarcin against different dinucleoside monophosphates proved that this protein is also a cyclizing RNase (Lacadena *et al.*, 1998, 1999), with an optimum pH of 5.0 (Pérez-Cañadillas *et al.*, 1998; Lacadena *et al.*, 1999). Therefore, ribotoxins follow the same general reaction scheme as the other members of the RNase T1 family. However, the catalytic efficiency of RNases T1 and U2 against naked RNA, homopolynucleotides or dinucleotides is several orders of magnitude higher. On the other hand, when assayed against natural substrates, ribotoxins cleave and consequently inactivate the ribosome with a second-order rate constant (k_{cat}/K_m of $1.7 \times 10^{10}\,M^{-1}\,s^{-1}$) that matches the catalytic efficiency of the fastest known enzymes (Korennykh *et al.*, 2006).

In the case of RNase T1, during the first step of the reaction Glu58 acts as a general base and His92 as a general acid (Figs 4 and 7). The hydrolysis of the cyclic derivative is catalyzed by the same groups, but their roles are reversed (Steyaert, 1997). In fact, the most common pair of catalytic residues found in microbial RNases is this Glu/His combination (Yoshida, 2001). Another His residue, His40, is required in its protonated form to assist the electrostatic stabilization of the transition state and, eventually, seems to be able to adopt the function of the general base, as shown

with Glu58-mutated variants of RNase T1 (Steyaert *et al.*, 1990; Steyaert, 1997). Superposition of the three-dimensional structures of RNases T1 and U2 with those of α-sarcin and restrictocin showed that the counterpart residues were α-sarcin's His137, Glu96, and His50, and restrictocin's His136, Glu95, and His49 (Fig. 4) (Sacco *et al.*, 1983; Martínez del Pozo *et al.*, 1988; Campos-Olivas *et al.*, 1996b). Production of wild-type and several mutant forms of restrictocin in *S. cerevisiae* showed that only the strain producing an H136L mutant was able to grow (Yang & Kenealy, 1992a, b). The same authors produced and partially characterized this same mutant in *A. niger* and *A. nidulans* (Brandhorst *et al.*, 1994), with very similar results. Not much later, another equivalent α-sarcin (H137Q) and restrictocin (H136Y) variants were isolated and characterized in detail from the structural and functional points of view; this confirmed that their lack of toxicity was due to the absence of ribonucleolytic activity, and not to major conformational changes (Kao & Davies, 1995; Lacadena *et al.*, 1995). Thanks to the production and further characterization of more mutants, affecting these three residues in α-sarcin and restrictocin, it is now well known that α-sarcin His137 and Glu96 are the only residues that are essential for performing the acid–base type reaction (Brandhorst *et al.*, 1994; Kao & Davies, 1995, 1999; Lacadena *et al.*, 1995, 1999; Sylvester *et al.*, 1997; Kao *et al.*, 1998); His50 would also contribute to the stabilization of the transition state but, in this case, would not be able to substitute for Glu96 as the general base in E96Q mutants (Fig. 7) (Lacadena *et al.*, 1999). This was inferred because substitution of His50 (or His49 in restrictocin) with different residues did not completely inactivate the enzyme, but rather decreased its k_{cat} values, so that it showed only residual enzymatic or cytotoxic activity, depending on the nature of the assay used (Nayak & Batra, 1997; Sylvester *et al.*, 1997; Lacadena *et al.*, 1999). In addition, it was proved that the three mentioned residues are required for the specific inactivation of the ribosomes, as each individual variant assayed, as well as the double and triple mutant versions, lacked this particular activity (Lacadena *et al.*, 1999).

The profile obtained for the pH dependence of the α-sarcin activity is typical for an acid–base catalyst but significantly different from that described for RNases T1 or U2 (Arima *et al.*, 1968a, b; Sylvester *et al.*, 1997; Pérez-Cañadillas *et al.*, 1998; Lacadena *et al.*, 1999). The α-sarcin H50Q mutant also shows quite different behavior, probably due to the absence of a positive charge in the Glu96 environment. Finally, whereas α-sarcin displays a low efficiency in hydrolyzing the cyclic intermediate, as most cyclizing RNases do, its H50Q variant is much more efficient in producing the 3′-AMP product at pH 7.0 (Lacadena *et al.*, 1999). The NMR measurements mentioned above were also used to calculate how these active site residues display p*K*a values far from their intrinsic values, which would explain these different behaviors in terms not only of specificity but also of pH dependence (Pérez-Cañadillas *et al.*, 1998; Lacadena *et al.*, 1999).

In the crystal complex of RNase T1 with the minimal substrate 3′-GMP (Loverix & Steyaert, 2001), Tyr38, Arg77 and Phe100 also appear to form the catalytic site of the enzyme. There has been speculation about these three residues, together with His40, forming a prearranged structural and dielectric microenvironment that is complementary in shape, charge and hydrogen-bonding capacity to the equatorial oxygens of the transition state, contributing to its optimal solvation/desolvation (Loverix & Steyaert, 2001). Tyr48, Arg121 and Leu145 are their three corresponding structural counterparts in α-sarcin (Figs 3 and 4), and therefore have been also studied.

RNase T1 Arg77 is located in the vicinity of the substrate phosphate moiety, but its potential functional role is not known, as all attempts to isolate any RNase T1 with a mutation affecting that residue have been unsuccessful (Steyaert, 1997). Thus, it has long been proposed that Arg77 of RNase T1 might facilitate the nucleophilic attack, but this has not been directly proven by site-directed mutagenesis (Steyaert, 1997). On the other hand, Arg121 of α-sarcin has been replaced by Gln or Lys, mutations that did not modify the conformation of the protein, but abolished its ribosome-inactivating activity. Unexpectedly, these mutants were still active against a small and nonspecific substrate such as ApA (similar K_m and lower catalytic efficiency than the wild-type protein) (Masip *et al.*, 2001). In addition, as mentioned above, the loss of the positive charge at that position produced dramatic changes in α-sarcin's ability to interact with phospholipid membranes (Masip *et al.*, 2001).

Regarding RNase T1 Phe100, a Leu residue (Leu145) occupies the equivalent position in α-sarcin (Figs 3 and 4). The side chain of Phe100 is an apolar catalytic element, stabilizing charge separations that occur in the transition state by controlling the dielectric environment (Doumen *et al.*, 1996). Characterization of an L145F variant of α-sarcin revealed that it was still an active RNase (the mutant exhibited a similar K_m and slightly lower catalytic efficiency against the ApA substrate), but displayed lower specificity than the wild-type protein against rRNA gene and SRL-like substrates (Masip *et al.*, 2003). Leu145 was also shown to be essential to preserve the electrostatic environment of the active site required to maintain the anomalously low p*K*a value reported for the catalytic His137 (Masip *et al.*, 2003).

One of the residues showing the largest NMR chemical shift variation in the L145F mutant of α-sarcin was Asn54, a conserved residue located in loop 2 (Fig. 3). It not only contributes to the high stability of ribotoxins, but is also required for their highly specific action on ribosomes, according to the results obtained after the characterization

of five different α-sarcins with mutations at this position (Siemer *et al.*, 2004). These results suggest that Asn54 is involved in local conformational arrangements at the substrate-binding pocket. The mutations at this position resulted in less efficient RNases, especially against nonspecific substrates such as poly(A). The RNase residues involved in the interaction with the base 5′ to the cleaved phosphodiester bond are usually referred as the base recognition residues. The results obtained with the α-sarcin Asn54 mutants are in agreement with the idea that residues 53–56 (52–55 in restrictocin) would form that recognition pocket in ribotoxins (Yang & Moffat, 1996). However, recognition of ribosomes involves a much more complex network of interactions, most of which would not be disturbed by the mutation of Asn54, which would explain why most of the mutants still retained the ability to specifically release the α-fragment (Siemer *et al.*, 2004). Overall, these results are in perfect agreement with the idea of local conformational rearrangements of the Asn54 and Leu145 mutants' active site, leading to less specific and less cytotoxic enzymes (Masip *et al.*, 2003; Siemer *et al.*, 2004). Another important observation was that ribotoxins lack a residue equivalent to RNase T1 Glu46, involved in discriminating guanine from adenine (Gohda *et al.*, 1994). The lack of this residue could explain why this ribotoxin is only purine-specific when assayed against naked RNA, and not guanine-specific, as is the case for RNase T1 (Endo *et al.*, 1983, 1988).

Residue Tyr48 in α-sarcin is conserved not only within the ribotoxin family, but also within the larger group of fungal extracellular RNases (Fig. 3) (Martínez-Ruiz *et al.*, 1999a, b). Tyr38 of RNase T1 forms a short hydrogen bond with one of the phosphate oxygens in the RNase T1/3′–GMP complex, an interaction that may be more favourable in the transition state (Loverix & Steyaert, 2001). A Y48F mutant of α-sarcin was shown to be inactive against polymeric RNA substrates, revealing the essential role of the OH group in the Tyr48 phenolic ring (Álvarez-García *et al.*, 2006). This mutant was, again, active against ApA, revealing that it retained ribonucleolytic activity at this level. In summary, the removed OH group only contributes slightly to the catalytic efficiency against ApA, but is essential for the characteristic ribotoxin activity (specific degradation of rRNA gene and SRL-like substrates).

Thus, Tyr48, Arg121 and Leu145 appear to be determinants of the ribotoxin activity of α-sarcin. Studies of the crystal structures of complexes of the α-sarcin-like ribotoxin restrictocin with inhibitors led to the proposal that these ribotoxins may use base flipping to enable cleavage at the correct site of the SRL substrates (Yang *et al.*, 2001). All studies so far suggest that these three residues would enable the base flipping performed by His50/Glu96/His137 that permits RNase cleavage at a unique phosphodiester bond (Yang *et al.*, 2001).

In addition, the N-terminal hairpin has been shown to modulate the catalytic activity of ribotoxins, in studies with different mutants of mitogillin, another ribotoxin with only a single substitution relative to restrictocin (Kao & Davies, 1999; 2000), and α-sarcin (García-Ortega *et al.*, 2001). These studies included deletion variants in which this hairpin had been eliminated without affecting the overall three-dimensional structure of the protein (García-Ortega *et al.*, 2002, 2005; García-Mayoral *et al.*, 2004). These mutants [α-sarcin Δ (7–22) and Aspf1 Δ (7–22)] retained their nonspecific ribonucleolytic activity as well as their ability to specifically cleave SRL-like oligonucleotides, but were not able to specifically inactivate rabbit ribosomes, and therefore were much less cytotoxic (García-Ortega *et al.*, 2002, 2005).

In conclusion, it is important to note the differences exhibited by HtA. As expected, because it is a ribotoxin, this protein caused the specific cleavage not only of rabbit 28S rRNA gene, but also of the SRL-like oligonucleotides used as substrates (Herrero-Galán *et al.*, 2007). However, when less specific substrates were employed, HtA showed quite distinct behavior, as reflected by the fact that it is not active against poly(A) but is active against poly(C) (Herrero-Galán *et al.*, 2007). This behavior must be linked to the structural differences displayed by HtA, but the interpretation is not obvious, as the behavior has also been observed with other wild-type and mutant ribotoxins (Nayak *et al.*, 2001). Most probably, this behavior reflects as yet unknown elements of the catalytic mechanism.

Interaction with the SRL and the ribosome

Ribosomes are different in terms of their components among the three phylogenetic domains, *Archea*, *Bacteria*, and *Eukarya*, but several functional regions are always conserved, probably because they are essential to preserve the protein biosynthesis machinery (Mears *et al.*, 2002; Uchiumi *et al.*, 2002). One of them is the SRL (Szewczak & Moore, 1995; Glück & Wool, 1996). This region is of particular interest, owing to its crucial role in elongation-related events in both prokaryotic and eukaryotic ribosomes. It contains the longest known universally conserved ribosomal sequence (A2654–A2665 in the *E. coli* 23S rRNA gene, and A4318–A4329 in the rat 28S rRNA gene), and shows a unique RNA shape, which is structurally preserved. It is so conserved that when the crystalline structure of the *Halobacterium marismortui* large ribosomal subunit was elucidated, the sequence of the 23S rRNA gene was fitted into the electron density map, nucleotide by nucleotide, starting from its SRL sequence (Ban *et al.*, 2000). This SRL is a distorted hairpin, with an unusually stiff central part, and a GAGA tetraloop, a G-bulged cross-strand A-stack, a flexible region, and a terminal A-form duplex (Fig. 2). It is

not associated with any deep electrostatic potential pockets of the ribosomes, and is not a major binding motif. However, together with the L11-binding region, the L7/L12 stalk, and the ribosomal proteins L6 and L14 (Figs 2 and 5c), it constitutes an elongation factor-binding site that is required for correct functioning of the ribosome (Endo & Wool, 1982; Cameron *et al.*, 2002; Van Dyke *et al.*, 2002). The L11-binding domain sequence is also universally conserved, in good agreement with its essential role (Mears *et al.*, 2002). Interestingly, the spatial orientation in the ribosome of both the SRL and the L11-binding domain varies not only among the different phyla (Ramakrishnan & Moore, 2001; Mears *et al.*, 2002; Uchiumi *et al.*, 2002), but also during the different steps of peptide bond formation (Gabashvili *et al.*, 2000). These differences might explain why different RIPs display different affinities when assayed against different ribosomal substrates (Schindler & Davies, 1977; Endo & Wool, 1982; Wool *et al.*, 1992; Uchiumi *et al.*, 2002). Mutations affecting the sequence contained in the SRL result in defective binding of elongation factors and aminoacyl-tRNA, as well as a decrease in translational fidelity (Liu & Liebman, 1996). Some of these mutations are lethal, reinforcing the importance of this region for the translational machinery (Leonov *et al.*, 2003). Studies on the dynamics and kinetics of the ribosome show considerable mobility of this region, known as the GTPase center, and its possible involvement in conformational changes essential for the correct performance of translation (Nilsson & Nissen, 2005).

Extensive studies using small synthetic oligoribonucleotides mimicking the SRL sequence (Correll *et al.*, 1998, 1999, 2003; Correll & Swinger, 2003) have shed light on the rRNA identity elements needed for ribotoxin recognition of the phosphodiester bond to be cleaved. These SRL analogs are indeed specifically recognized and cleaved by the ribotoxins (Endo *et al.*, 1988; Kao *et al.*, 2001), although larger amounts of enzyme need to be employed, as mentioned in the previous section, indicating that the recognition is not as specific as with the whole ribosome. Unquestionably, they have been of great help, because they do maintain the structural features of the SRL within the complete ribosome and have been used to establish the structural determinants needed for the recognition between the SRL and ribotoxins. Thus, docking models and kinetic experiments were used to predict rRNA and protein regions capable of establishing interactions with the ribotoxins (Yang & Moffat 1996; Pérez-Cañadillas *et al.*, 2000; Correll *et al.*, 2004; García-Mayoral *et al.*, 2005b). Some of these predictions were confirmed by the determination of the crystal structures of restrictocin–inhibitor complexes made with several SRL-like RNA oligo variants (Fig. 5a) (Yang *et al.*, 2001). These studies included the resolution of the structures of two mutant versions of the oligonucleotides that mimic the 28S rRNA gene SRL motif (Correll *et al.*, 2003), as well as of three other different SRL analogs in complex with restrictocin (Yang *et al.*, 2001). According to these results, there are two SRL areas that are recognized by both toxins and elongation factors, the GAGA tetraloop and the bulged G2655 (Fig. 2) (Moazed *et al.*, 1988; Glück & Wool, 1996; Munishkin & Wool, 1997; Pérez-Cañadillas *et al.*, 2000). Thus, G2655 represents the most critical site for binding of elongation factors (Munishkin & Wool, 1997). However, the primary determinant of recognition does not seem to be the nucleotide type, but rather the SRL conformation (Munishkin & Wool, 1997; Correll *et al.*, 1999, 2003). Molecular dynamics simulation of two SRL structures based on crystal structures of *E. coli* and rat SRL motifs revealed that the GAGA tetraloop is the most dynamic part of this motif (Špačková & Šponer, 2006). In fact, GNRA tetraloops adopt an unfolded geometry upon binding of elongation factors and/or toxins, as was observed in the above-mentioned SRL–restrictocin complexes (Yang *et al.*, 2001). It has already been mentioned that these studies led to the proposal that ribotoxins may use base flipping to enable cleavage at the correct site of the SRL substrates (Yang *et al.*, 2001), and that this base flipping may be a common cleavage mechanism for endonucleases acting on folded substrates, as is the case for ribotoxins (Yang *et al.*, 2001). In summary, according to the above-mentioned results, two distant regions of the ribotoxin molecules participate in their specific interaction with the SRL. The Lys-rich region corresponding to loop 3 of α-sarcin interacts with the negatively charged phosphodiester bond around the bulged G, and the sequence stretch of loop 2, comprising residues 51–55 (a sequence stretch that includes the mentioned Asn54) and some residues from loop 5, contacts the conserved GAGA tetraloop that contains the sequence cleaved by the toxin (Fig. 5s).

It is, however, quite obvious that these interactions with the SRL do not by themselves explain the exquisite specific activity displayed by ribotoxins against intact ribosomes. Consequently, some other interactions with additional ribosomal elements are required. In this regard, it has recently been shown that the ribosomal context enhances the reaction rate by several orders of magnitude. This catalytic advantage seems to arise from favorable electrostatic interactions with the ribosome (Korennykh *et al.*, 2006). The positively charged ribotoxins bind with high affinity and speed, thereby enhancing the rate of SRL cleavage by several orders of magnitude, matching the catalytic efficiency of the fastest known enzymes (Korennykh *et al.*, 2006). α-Sarcin's surface, for example, is highly charged: 39% of the surface is composed of charged side chains and 26% of polar side chains (Pérez-Cañadillas *et al.*, 2000). Therefore, these results suggest a mechanism of target location whereby α-sarcin encounters ribosomes randomly and diffuses within the ribosomal electrostatic field to the SRL. Long ago, it was

reported not only that the ribonucleolytic activity of α-sarcin was completely inhibited by NH_4^+, K^+, or Na^+ at concentrations higher than 0.2 M, as well as by millimolar levels of some divalent cations such as Ca^{2+}, Mn^{2+}, or Mg^{2+} (Endo *et al.*, 1983; Martínez del Pozo *et al.*, 1989), but also that it binds Zn^{2+}, Cd^{2+}, and Co^{2+}, with an affinity corresponding to dissociation constants in the millimolar range (Martínez del Pozo *et al.*, 1989). This binding was proposed to be mediated by interactions with the active site His side chains, and affected fluorescence emission, most probably modifying the microenvironment of Trp51. Whereas in these studies it was shown that Zn^{2+} cations were effective inhibitors of its ribonucleolytic activity, it was also shown that the inhibition promoted by most of the other cations studied was due to the establishment of interactions with the substrates used, rather than to the existence of a specific interaction with the protein (Martínez del Pozo *et al.*, 1989).

Within this context, it must be considered that internal motions allow recognition elements to screen a significant part of the conformational space, increasing the chances of successful binding. As explained earlier, residues 1–26 in α-sarcin form a long β-hairpin that can be considered as two consecutive minor β-sheets connected by a hinge region. The second β-sheet, coincident with residues 7–22, is one of the regions with the highest conformational flexibility, appearing to be folded independently from the protein core (Pérez-Cañadillas *et al.*, 2000, 2002; García-Mayoral *et al.*, 2004). The results obtained with the previously mentioned Δ (7–22) α-sarcin mutant suggested that this protein would interact with the ribosome in at least two regions, i.e. the well-known SRL of the rRNA gene, and a different region recognized by the β-hairpin of the protein (García-Ortega *et al.*, 2002). Its three-dimensional structure in solution (García-Mayoral *et al.*, 2004) showed that the folding of wild-type α-sarcin was preserved, including the spatial conformation of the loops. The most significant differences were concentrated in loop 2, the new orientation of loop 3, and the dynamics of loop 5, where conformational heterogeneity was observed as a consequence of the removal of important interactions with residues in the native motif (García-Mayoral *et al.*, 2004). Thus, its structural integrity and ability to specifically cleave SRL-like oligos was preserved, but the α-sarcin-specific recognition of the ribosome disappeared. Modeling the highly specific ribotoxin recognition of ribosomes using three-dimensional structures of wild-type and Δ(7–22) α-sarcins suggested two more hitherto unidentified interactions (García-Mayoral *et al.*, 2005b). One of them would occur between a short sequence stretch of the α-sarcin loop 2 and the ribosomal protein L6 (Fig. 5c). The second would occur between the residues corresponding to the deleted distal part of the β-hairpin and protein L14 (Fig. 5c). These two prokaryotic ribosomal proteins are immediate neighbors of the SRL (Fig. 2a), are present in the three living phyla (Ban *et al.*, 2000), and seem to undergo the most substantial changes, according to the X-ray and cryo-electron microscopy maps of the ribosome (Ban *et al.*, 2000; Gabashvili *et al.*, 2000). Obviously, the interaction involving the β-hairpin would not be possible for the deletion mutant, and appears to be crucial for the specific ribosome recognition (García-Mayoral *et al.*, 2005b). This hypothesis was reinforced by the observation that a region homologous to the 11–16 sequence of α-sarcin can be found in (EF-2) from *S. cerevisiae* (Kao & Davies, 1999; García-Mayoral *et al.*, 2005b). Both groups of polypeptides, ribotoxins and ribosomal proteins L14 (and their L23 counterparts in eukaryotic organisms), represent families of highly homologous proteins. The interacting regions of α-sarcin and L14 are conserved but show some degree of variability (García-Mayoral *et al.*, 2005b), especially regarding the L14 residues involved (L23 proteins have sequences that are only distantly related). This fact helps to explain not only the extraordinary catalytic efficiency of ribotoxins against ribosomes, but also their different potencies, depending on the origin of the ribosome assayed (Schindler & Davies, 1977; Endo & Wool, 1982; Endo *et al.*, 1983; García-Mayoral *et al.*, 2005b).

Ribotoxins as natural killers of intact cells

It has already been explained how α-sarcin is able to inactivate ribosomes in cell-free systems of a great variety of organisms (Endo *et al.*, 1993a, b; Kao & Davies, 1995), but it displays marked selectivity when intact cells are used as targets. This specificity seems to be determined by its ability to penetrate cells. Thus, α-sarcin is active against transformed or virus-infected mammalian cells, in the absence of any other permeabilizing agent (Fernández-Puentes & Carrasco, 1980; Carrasco & Esteban 1982; Olmo *et al.*, 1993, 2001; Turnay *et al.*, 1993; Stuart & Brown, 2006). The protein was also cytotoxic, inhibiting protein biosynthesis, when assayed against eight different human and rat tumor cell lines of mesenchymal, glial or epithelial origin (Turnay *et al.*, 1993): This effect was saturable and consistent with passage across the cell membrane being the rate-limiting step, but no membrane damage or mitochondrial activity alterations were detected (Turnay *et al.*, 1993). Again, these experiments confirmed that α-sarcin exhibits an intrinsic and rather specific cytotoxic character, in the absence of any external permeabilizing agent, virus included, when assayed against some transformed cell lines. The particular reasons for this selectivity at the molecular level have not been completely established yet; however, as mentioned above, the presence of acidic phospholipids on the outer leaflet of the membrane seems to be one of the determining factors (Connor *et al.*, 1989; Gasset *et al.*, 1989, 1990; Zachowski, 1993).

The mechanism of internalization of α-sarcin into intact human rhabdomyosarcoma cells and the cellular events resulting in the induction of cell death have been studied (Olmo *et al.*, 2001). According to these results, the toxin is internalized via endocytosis involving acidic endosomes and the Golgi, as deduced from the ATP requirement and the effects of NH_4Cl, monensin and nigericin on its cytotoxicity. In addition to the specific cleavage of 28S RNA associated with protein biosynthesis inhibition, α-sarcin killed rhabdomyosarcoma cells via apoptosis. This apoptosis was not just a general direct consequence of protein biosynthesis inhibition, as deduced from a comparative analysis of the effects of α-sarcin and cycloheximide (Olmo *et al.*, 2001). Furthermore, experiments with a catalytically inactive α-sarcin mutant (H137Q), which is neither toxic nor apoptotic, revealed that it was directly related to the catalytic effects of the toxin on the ribosomes, as this mutant displays identical lipid-interacting abilities to those of the wild-type protein (Lacadena *et al.*, 1995).

The loss of the positive charge at the position corresponding to α-sarcin Arg121 produced a dramatic impairment of its ability to interact with phospholipid membranes (Masip *et al.*, 2001), supporting the conclusion that Arg121 is a crucial residue for the characteristic cytotoxicity of α-sarcin and presumably of the other fungal ribotoxins. In agreement with their altered ribonucleolytic and lipid-interaction activities, all of the mutants studied with mutations affecting the enzymatic specificity of the protein, especially the deletion Δ(7–22) mutant, showed diminished cytotoxic effects on human rhabdomyosarcoma cells (García-Ortega *et al.*, 2002). Even restrictocin shows a lower phospholipid-interacting ability, which is correlated with decreased cytotoxicity (García-Ortega *et al.*, 2001), as mentioned earlier in this review. On the other hand, HtA shows a very similar cytotoxic potency, in terms of IC_{50} (concentration required to produce 50% of protein biosynthesis inhibition) (Herrero-Galán *et al.*, 2007).

Ribotoxins as allergens

Fungi represent one of the principal sources of allergens. *Aspergillus fumigatus*, a human pathogenic species, has been well studied as an opportunistic pathogen (Walsh & Pizzo, 1988; Bodey & Vartivarian, 1989). Invasive infection is usually fatal unless treated early, and even then, antifungal therapy is often unsuccessful. The incidence of fungal infections has risen lately, owing to the increase in the number of immunocompromised patients, and *A. fumigatus* infection is common postoperatively (Pasqualotto, 2006). A link between fungal allergy and severe asthma is generally accepted, but is still poorly understood (Ronning *et al.*, 2005; Denning *et al.*, 2006). Among the reasons why *A. fumigatus* can behave as a human pathogen is its ability to grow quickly at temperatures as high as 50 °C, it being the most thermophilic of the *Aspergillus* spp. (Ronning *et al.*, 2005); all the other known ribotoxin-producing fungi can scarcely grow when cultured above 30 °C. It is also remarkable that *A. fumigatus* is a more common source of allergy and asthma than either *A. nidulans* or *A. oryzae*, the other two *Aspergillus* spp. whose genome sequences have been determined (Galagan *et al.*, 2005; Machida *et al.*, 2005; Nierman *et al.*, 2005). Interestingly, all *A. fumigatus* allergens have close homologs in the other two species, with the exception of the ribotoxin Aspf1 and the metalloprotease Aspf5 (Ronning *et al.*, 2005), but it is not yet clear whether Aspf1 is a critical factor in triggering an allergic response. In this regard, it should be remembered that allergens are usually identified as those proteins recognized by the IgE antibodies contained in the sera of allergic patients. In relation to this, restrictocin was also found in the urine of patients with disseminated aspergillosis (Arruda *et al.*, 1990, 1992; Lamy *et al.*, 1991), and antibodies have been also used to prove that it accumulates in the vicinity of nodes of fungal infection (Lamy *et al.*, 1991). Although it has been proven that Aspf1 is not a major virulence factor in *A. fumigatus* infections (Paris *et al.*, 1993; Smith *et al.*, 1993, 1994), this protein is clearly involved in the pathogenicity of allergic bronchopulmonary aspergillosis (ABPA), the most severe form of allergic inhalant diseases, as these patients show high levels of Aspf1-specific IgE (Kurup *et al.*, 1994; García-Ortega *et al.*, 2005). ABPA has a prevalence of 1–2% in patients with persistent asthma, but this value increases to 15% in cystic fibrosis patients (Greenberger, 2002; Kurup *et al.*, 2006). The explanation for *A. fumigatus* being the mold usually involved in these diseases seems to be, again, its ubiquity and its small spores, which grow optimally at 37 °C. Thus, it can colonize the respiratory tract of the host, leading to the onset of the pathologic events (Banerjee & Kurup, 2003).

Extracts of *A. fumigatus* are frequently used to diagnose allergic reactions, but they are highly complex mixtures, containing up to 200 different proteins and glycoproteins and low molecular weight compounds (Piechura *et al.*, 1983), and are very difficult to standardize. Attempts to improve diagnosis are focusing on the employment of homogeneous standard preparations of recombinantly produced allergens (Crameri *et al.*, 1998; Kurup *et al.*, 2006). In this regard, it is therefore important to point out that Aspf1 was the first recombinant allergen tested *in vivo* (Moser *et al.*, 1992), and showed complete concordance with serologic determinations (Moser *et al.*, 1992; Crameri *et al.*, 1998; Hemmann *et al.*, 1999). Unfortunately, the recombinant native allergen is not devoid of cytotoxic activity, and can trigger anaphylaxis.

As mentioned before, ribotoxins have much longer loops than the other nontoxic fungal RNases, which are supposedly involved in their specificity, toxicity and antigenicity.

Once again, it must be noted that the Δ(7–22) region, which contains the ribotoxin-characteristic N-terminal β-hairpin, shows the highest amino acid sequence variability among ribotoxins (Fig. 2) (Martínez-Ruiz *et al.*, 1991a, b, 2001), and is highly flexible and exposed (Pérez-Cañadillas *et al.*, 2000; García-Mayoral *et al.*, 2004). Indeed, Aspf1 differs from α-sarcin in only 19 (87% sequence identity) residues, but five of these 19 amino acid differences are located at this N-terminal β-hairpin. As it is generally assumed that the exposed and highly flexible regions are usually good candidates to be B-cell epitopes in proteins, this β-hairpin could certainly be a major determinant of the immunoreactivity of these proteins. This was confirmed through the production and characterization of Aspf1, α-sarcin, and their corresponding Δ(7–22) variants (García-Ortega *et al.*, 2002, 2005). First, these data confirmed the significant prevalence of Aspf1-specific IgE antibodies in sera from patients sensitized to *Aspergillus* (García-Ortega *et al.*, 2005), as reported before by other authors (Kao *et al.*, 2001; Greenberger, 2002; Kurup *et al.*, 2006). This result was particularly important in the ABPA patients studied, as anti-Aspf1 IgE antibodies were detected in 100% of them. Second, and although several earlier studies with synthetic peptides overlapping the above-mentioned region produced controversial results regarding its antigenic behavior (Kurup *et al.*, 1998, Madan *et al.*, 2004), the three proteins studied, Aspf1Δ (7–22), α-sarcin, and α-sarcin Δ (7–22), showed marked decreases in their reactivity to Aspf1 IgE antibodies (García-Ortega *et al.*, 2005), indicating that the deleted portion was involved in at least one allergenic epitope. However, although important, this cannot be the only allergenic epitope within this molecule, as deduced from enzyme-linked immunosorbent assay (ELISA) inhibition experiments. The essential residues for epitopes in Aspf1 are changed in wild-type α-sarcin, as inferred from the fact that the response against the sera of the patients was even lower for the latter than for the Aspf1Δ (7–22) mutant. Despite this decreased IgE reactivity, the prevalence of the three Aspf1 variants remained essentially unaffected, and they retained most of the IgG epitopes (García-Ortega *et al.*, 2005). Thus, these noncytotoxic deletion variants of ribotoxins are promising molecules for use in immunomodulating therapies for *Aspergillus* hypersensitivity and diagnosis. However, *in vivo* assays are still required to assess this possibility.

Immunotoxins

One of the goals of antitumor therapy is the preparation of immunotoxins according to the idea described by Ehrlich in 1906, who introduced the concept of targeting cancer cells with a 'magic bullet' consisting of a tissue-specific carrier that would deliver toxic agents to neoplastic tissue (Ehrlich, 1956). In the past decade, an increased amount of clinically oriented research involving immunotoxins has been published (Reiter & Pastan, 1998; Kreitman *et al.*, 1999; Brinkmann, 2000; Li *et al.*, 2004). Immunotoxins are therapeutic agents with a high degree of specificity, composed of targeting moieties, such as antibodies or physiologically important ligands (such as growth factors or cytokines), linked to toxins, chiefly toxic proteins from plants or bacteria (Brinkmann & Pastan, 1994; Reiter & Pastan, 1998; Kreitman, 2001).

Initially, immunotoxins were prepared by conjugating toxins to monoclonal antibodies. The targeting moiety of these first-generation immunotoxins was the whole antibody molecule (Kreitman, 2000). As the binding sites for antigen are on the variable regions of antibodies, further studies were performed to verify that Fab fragments, obtained after IgG papain digestion, retained the ability to interact with antigens (Ward *et al.*, 1989; Wörn & Plückthun, 2001), leading to the so-called Fab or Fv immunotoxins, which were easily internalized because of their smaller size (Brinkmann, 2000). The development of advanced technologies allowed the production of recombinant immunotoxins, stabilized by a flexible peptide (scFv) or by a disulfide bridge between the variable domains (dsFv), that can be expressed in several model organisms, are easily modified by genetic engineering, and are more stable (Kreitman, 2003; Li *et al.*, 2004).

Regarding the toxin moiety, the most representative toxins employed have been ricin from plants and *Pseudomonas* exotoxin A (PE) or diphtheria toxin (DT) from bacteria. Ricin is composed of two subunits linked together by a disulfide bond, chain A being responsible for the glycosidase activity, leading to the inactivation of ribosomes (Olsnes & Pihl, 1973a, b; Endo *et al.*, 1987), and is the one usually used to make immunotoxins (Ghetie *et al.*, 1993; Engert *et al.*, 1997; Schnell *et al.*, 1998). Ricin depurinates a single nucleotide contiguous to the phosphodiester bond cleaved by ribotoxins (Endo & Tsurugi, 1987; Endo *et al.*, 1987), a catalytic action that renders the ribosome inactive too. Different immunotoxins have been obtained that contain the whole blocked ricin or deglycosylated chain A (Pastan *et al.*, 1992; O'Toole *et al.*, 1998). Regarding bacterial toxins, PE and DT are single-chain proteins that inhibit protein synthesis by ADP-ribosylating EF-2 (Carroll & Collier, 1987). Among PE- and DT-based immunotoxins, the most commonly used involve truncated versions of the toxins, produced by genetic excision of their binding domain, resulting in PE38 or PE40 variants (Kondo *et al.*, 1988; Kreitman *et al.*, 1990, 1993; Pastan, 2003), and DT388 or DT389 variants (Foss *et al.*, 1998; LeMaistre *et al.*, 1998), respectively.

Ribotoxins have several advantages for use in the design of immunotoxins, namely, their small size, high thermostability, resistance to proteases, and highly efficient

ribonucleolytic activity (Gasset *et al.*, 1994; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). Poor immunogenicity and low toxicity in mice have also been described in relation to restrictocin (Rathore & Batra, 1996). Thus, different ribotoxins have been used as components of immunotoxins (Orlandi *et al.*, 1988; Conde *et al.*, 1989; Hertler & Frankel, 1989; Wawrzynczak *et al.*, 1991; Better *et al.*, 1992; Rathore & Batra, 1996; 1997a, b; Rathore *et al.*, 1997). Initially, ribotoxin-based immunotoxins were constructed by chemical conjugation, as described for, among others, mitogillin (Better *et al.*, 1992), restrictocin (Orlandi *et al.*, 1988; Conde *et al.*, 1989; Rathore & Batra, 1996) and α-sarcin (Wawrzynczak *et al.*, 1991). Second-generation immunotoxins were later designed, mostly related to restrictocin single-chain immunotoxins produced by fusing restrictocin cDNA with that encoding the scFv region of the monoclonal antibody directed to the human transferrin receptor (anti-TFR) (Rathore & Batra, 1997a, b), joined by a linear flexible peptide to promote the independent folding of the two immunotoxin moieties. These constructions were further engineered to enhance the intracellular processing and delivery of restrictocin (Goyal & Batra, 2000).

A few immunotoxins containing α-sarcin have been described (Wawrzynczak *et al.*, 1991; Rathore *et al.*, 1997), with α-sarcin chemically coupled to anti-TFR or anti-Fib75. The α-sarcin used in these constructions was obtained either from *A. giganteus* cultures (Wawrzynczak *et al.*, 1991) or from heterologous expression in *E. coli* cultures (Goyal & Batra, 2000). Promising results were obtained when cytotoxicity was measured, with IC_{50} values similar to those obtained with plant or bacterial toxin-based immunotoxins (Goyal & Batra, 2000). The α-sarcin immunotoxin showed equal stability and specific activity on the target cells and similar pharmacokinetics to those of analogous immunotoxins (Wawrzynczak *et al.*, 1991). However, further studies with α-sarcin-based immunotoxins, including clinical trials, were not performed, probably because of the large size of the immunotoxin, which could hinder correct internalization in solid tumors, or because of the low structural stability of the immunoconjugates. It must be noted that these α-sarcin immunotoxins were not made as recombinant second-generation immunotoxins, such as the single-chain immunotoxins (scFv-IMTX) described later for restrictocin (Rathore & Batra, 1997a, b), which gave better results in terms of stability and cytotoxicity assays *in vivo*. Moreover scFv-IMTX can be easily modified by genetic engineering to improve the cytotoxic activity or to diminish unspecific toxicity *in vivo* or immunogenicity.

In relation to this, a single-chain immunotoxin has been recently produced in the methylotrophic yeast *P. pastoris*, composed of the variable domains of the B5 monoclonal antibody, specific against $Lewis^Y$ carbohydrates, which are very abundant in carcinomas, bound to α-sarcin through a peptide containing a furin cleavage site (scFv-IMTXαS) (Lacadena *et al.*, 2005). *Pichia pastoris* has emerged as a robust heterologous expression host, owing to the efficient secretory expression of complex recombinant proteins with correct intramolecular and intermolecular disulfide bonds that do not require additional *in vitro* unfolding and refolding strategies, unlike most immunotoxins that are heterologously expressed in bacteria (Cregg *et al.*, 1993; Gurkan & Ellar, 2003, 2005). Indeed, *P. pastoris* possesses tightly regulated promoters, such as that of the *alcohol oxidase 1* gene (AOX1), which is uniquely suited for the controlled expression of foreign genes (Cregg *et al.*, 1989). Thus, several immunotoxins have been successfully produced extracellularly in *P. pastoris* (Woo *et al.*, 2002, 2004, 2006; Lacadena *et al.*, 2005; Liu *et al.*, 2005).

The monoclonal antibody (mAb) B5 belongs to a family of mAbs directed against a $Lewis^Y$-related carbohydrate antigen that is overexpressed on the surface of many carcinomas, including breast and colon solid tumors (Pastan & Fitzgerald, 1991). Different members of the family have been used as the targeting moiety in many immunotoxins, such as mAb B3 (Brinkmann *et al.*, 1991, 1993; Pai *et al.*, 1991, 1996; Benhar & Pastan, 1995a; Bera & Pastan, 1998), mAb B1 (Pastan & Fitzgerald, 1991; Benhar & Pastan, 1995b; Kuan & Pastan, 1996), and mAb B5 (Benhar & Pastan, 1995a, b). Indeed, mAb BR96 and mAb 3S193 have also been evaluated for targeted immunotherapy (Trail *et al.*, 1993; Rosok *et al.*, 1998; Scott *et al.*, 2000). At least three of these immunotoxins or immunoconjugates have recently been evaluated in phase I trials in patients with cancer, with promising results (Pai *et al.*, 1996; Brinkmann, 2000).

scFv-IMTXαS produced in *P. pastoris* displays the characteristic ribonucleolytic activity of α-sarcin and specific cytotoxicity against targeted cell lines containing the $Lewis^Y$ antigen (Lacadena *et al.*, 2005). Furthermore, studies on the characterization of genetically engineered immunotoxins based on that mentioned above, with increased stability and affinity, are being performed (Lacadena *et al.*, 2005).

Conclusions and future prospects

Ribotoxins are unique RNases in terms of specificity and cytotoxicity. Their remarkable and exquisitely specific ribonucleolytic action, as well as their innate ability to cross membranes, have been subjects of study for many years, and are now quite well understood in molecular terms, through the combination of a wide variety of structural, spectroscopic, biochemical, and cellular techniques, together with the production and characterization of a large number of mutants (Lamy *et al.*, 1992; Gasset *et al.*, 1994; Wool, 1997; Kao *et al.*, 2001; Martínez-Ruiz *et al.*, 2001). The determination of several high-resolution ribosomal structures and the

use of different lipid model vesicles and transformed cell lines, susceptible or not to the action of these toxins, have been of great help in deciphering the details of the cytotoxic mechanism of ribotoxins at the molecular level. The existence of very similar and well-known noncytotoxic fungal extracellular RNases, such as RNase T1, has been also very useful. Presumably, these RNases are noncytotoxic because they lack the above-mentioned ability to cross a phospholipid bilayer. Thus, they have been, and still are, excellent reference models with which to approach the study of the behavior of this family of proteins. Unfortunately, the natural function of ribotoxins still remains unknown, and it is definitively one of the most interesting questions that needs to be answered. In this regard, studies on the regulation of ribotoxin production within the context of their natural environment are still required. In fact, much indirect evidence suggests that these proteins are synthesized under a variety of stress conditions (Olson *et al.*, 1965b; Meyer & Stahl, 2002, 2003; Meyer *et al.*, 2002), but further direct characterization of these mechanisms and their regulation is still needed. In relation to this, the eventual functional connection with the prokaryotic TA systems (Condon, 2006) must not be dismissed. Indeed, the discovery and characterization of HtA, a singular ribotoxin from the structural and functional points of view, has opened a new door to the acquisition of additional clues about the origin and functionality of fungal ribotoxins. Further characterization of this protein and similar ones, which will eventually be discovered, given the increasingly rapid discovery of new ribotoxins, will greatly improve our understanding of ribotoxin action in the natural context, mostly involving the filamentous fungi.

This lack of knowledge about their natural function does not, however, preclude their employment as therapeutic agents. Despite the fact that their use as antitumor agents was abandoned early, due to high toxicity (Roga *et al.*, 1971), it is also true that the actual accumulation of data about their mechanism of action allows the optimistic view that these ribotoxins, or probably some modified variants of them, might be used with therapeutic aims. In relation to this, the production of hypoallergenic mutants and immunotoxins stand out as the most feasible alternatives in the mid-term future.

Regarding the first approach, it must be remarked how *Lactococcus lactis*, a primary constituent of many industrial and artisanal starter cultures used for the manufacture of a wide range of fermented dairy products, has been exploited through applications as a cell factory for metabolite and membrane protein production and as a delivery system for therapeutic molecules in the gastrointestinal tract (Steidler *et al.*, 2000, Kunji *et al.*, 2005). The status of *L. lactis* as a 'generally regarded as safe' (GRAS) organism confers this system with the features required to try immunotherapeutic protocols for Aspf1-related allergic diseases. In relation to this, *L. lactis* strains capable of secreting the above-mentioned hypoallergenic variants of Aspf1 have been obtained (Alegre-Cebollada *et al.*, 2005; García-Ortega *et al.*, 2005), and although their use as potential delivery systems has yet to be tested, they constitute one of the research directions that should be immediately explored.

Immunotoxins are another promising alternative for the employment of ribotoxins as therapeutic agents against tumorigenic processes. The production in large amounts of optimized immunotoxin versions of α-sarcin (Lacadena *et al.*, 2005) and other microbial RNases is well under way, and it is definitively one of the research paths to be followed in the near future.

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Objetivos

En esta Tesis Doctoral se ha pretendido ahondar en el conocimiento de las ribotoxinas fúngicas y de algunas de sus posibles aplicaciones clínicas, abordando su estudio desde diferentes puntos de vista para alcanzar los siguientes objetivos:

- Estudio de las relaciones estructura-función de las ribotoxinas, en lo referente a:

  - Complementar el estudio del centro activo de esta familia de proteínas. Para ello, se planteó el análisis de la función del único residuo conservado en el centro activo de la α-sarcina cuyo papel aún no se había determinado. Este estudio se completó con el análisis de residuos clave para la actividad ribonucleolítica de la ribonucleasa microbiana no tóxica más próxima filogenéticamente a las ribotoxinas, la RNasa U2.

  - Avanzar en el análisis del elemento de estructura más característico de las ribotoxinas, la horquilla β del extremo amino-terminal, en cuanto a su función de reconocimiento del sustrato y de interacción con membranas celulares.

  - Elucidar el efecto de la acción de las ribotoxinas en la funcionalidad del ribosoma.

- Sentar las bases iniciales de algunas aplicaciones clínicas de las ribotoxinas. En concreto:

  - Desarrollar un modelo de ratón con hipersensibilidad a Asp f 1 para poder evaluar *in vivo* la hipoalergenicidad de algunos mutantes de α-sarcina y Asp f 1.

  - Desarrollar cepas de *Lactococcus lactis* productoras de α-sarcina, Asp f 1 y mutantes con afinidad reducida por IgE para emplearlas como vehículo de administración oral de estas proteínas.

Resultados

Resultados A

RELACIONES ESTRUCTURA-FUNCIÓN EN LAS RIBOTOXINAS

A1. La tirosina 48, un residuo conservado de las ribotoxinas, está implicada en la degradación del RNA por parte de la α-sarcina.

En el centro activo de la ribonucleasa T1, junto al par ácido/base (His 92 y Glu 58, respectivamente) y a la His 40, aparece un grupo de residuos que contribuyen a la estabilización del estado de transición de la reacción: la Tyr 38, la Arg 77 y la Phe 100. Sus equivalentes en la α-sarcina son la Tyr 48, la Arg 121 y la Leu 145. Estos dos últimos residuos ya fueron estudiados anteriormente, comprobándose su implicación en la catálisis específica de las ribotoxinas. Pero la Tyr 48, a pesar de ser un residuo conservado no sólo dentro de la familia de las ribotoxinas sino en todo el grupo de ribonucleasas extracelulares fúngicas, nunca había sido objeto de estudio. En este trabajo se muestra cómo se produjo y purificó a homogeneidad un mutante de la proteína en el que esa tirosina está sustituida por fenilalanina. El análisis espectroscópico, que incluyó dicroísmo circular, emisión de fluorescencia y resonancia magnética nuclear, demostró que el mutante mantenía la conformación de la proteína nativa. Tampoco se detectaron diferencias en cuanto a la estabilidad térmica de la proteína silvestre, ni a su capacidad de interacción con vesículas de fosfolípidos. En cambio, los estudios enzimáticos revelaron el papel esencial del grupo –OH del anillo fenólico de la Tyr 48. La sustitución mencionada da como resultado una proteína que, aunque mantiene la capacidad para romper el enlace fosfodiéster de un sustrato mínimo como el dinucleósido ApA, carece totalmente de actividad ribonucleolítica frente a sustratos poliméricos de RNA, incluidos los ribosomas, sustrato natural de las ribotoxinas.

Trabajo A1: **Álvarez-García E**, García-Ortega L, Verdún Y, Bruix M, Martínez del Pozo Á y Gavilanes JG (2006) Tyr-48, a conserved residue in ribotoxins, is involved in the RNA-degrading activity of α-sarcin. Biol Chem. 387: 535-541.

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Tyr-48, a conserved residue in ribotoxins, is involved in the RNA-degrading activity of α-sarcin

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Abstract

Residue Tyr-48 in α-sarcin is conserved not only within the ribotoxin family, but also within the larger group of extracellular fungal ribonucleases, best represented by RNase T1. A mutant protein in which this Tyr residue was substituted by Phe has been produced and isolated to homogeneity. It was spectroscopically analyzed by means of circular dichroism, fluorescence emission and NMR. Taken together, these results and those from enzyme characterization have revealed the essential role of the -OH group from the Tyr-48 phenolic ring in the cleavage of polymeric RNA substrates, including the ribosome-embedded 28S rRNA, the natural substrate of ribotoxins. Thus, the mutant protein does not degrade its natural ribosomal RNA substrate. However, it has been shown that this Y48F mutant still retains its ability to cleave a phosphodiester bond in a minimal substrate such as the dinucleoside phosphate ApA. The role of different α-sarcin residues within the enzyme reaction catalyzed by this protein is discussed.

Keywords: α-sarcin; catalytic tyrosine; ribonuclease; RNase T1.

Introduction

Fungal ribotoxins are a group of highly specific ribonucleases (RNases) that display cytotoxic activity by first entering cells and then inactivating ribosomes (Kao et al., 2001; Martínez-Ruiz et al., 2001), promoting cell death by apoptosis (Olmo et al., 2001). This effect results from the cleavage of a single and unique phosphodiester bond within a universally conserved sequence of the larger rRNA, known as the sarcin/ricin loop (SRL) (Schindler and Davies, 1977; Endo and Wool, 1982; Endo et al., 1983). Ribotoxins share a high degree of sequence identity and the same specific mechanism of action (Wool, 1997; Kao et al., 2001; Martínez-Ruiz et al., 2001). α-Sarcin from *Aspergillus giganteus* and restrictocin from *Aspergillus restrictus*, the best-characterized ribotoxins, display 85% sequence identity over 150 residues (Sacco et al., 1983; Martínez-Ruiz et al., 1999a,b; García-Ortega et al., 2001) and almost identical three-dimensional structure (Yang and Moffat, 1996; Pérez-Cañadillas et al., 2000). Many other proteins of this family have been isolated and studied (Kao et al., 2001; Martínez-Ruiz et al., 1999a, 2001) and, although they have not been as well characterized, it is now clear that ribotoxins can be considered to belong to the larger family of fungal secreted RNases, usually represented by RNase T1 (Steyaert, 1997). All of them share a common structural fold (Yang and Moffat, 1996; Lacadena et al., 1998; Pérez-Cañadillas et al., 2000) (Figure 1) and are cyclizing RNases (Steyaert, 1997; Lacadena et al., 1999), although most of them are not cytotoxic and exhibit different ribonucleolytic activities. Based on the details of reported structure-activity relationships, RNase T1 is considered a model enzyme. The RNase T1-catalyzed reaction involves a general base/general acid pair (Glu-58 and His-92, respectively) and an electrostatic catalyst (His-40) (Steyaert, 1997; Zegers et al., 1998; Loverix and Steyaert, 2001). These residues have structurally equivalent counterparts in ribotoxins, corresponding to α-sarcin Glu-96/His-137 and His-50, respectively (Figure 1), and mutation studies have already revealed their involvement in catalysis by α-sarcin (Lacadena et al., 1999). Tyr-38, Arg-77 and Phe-100 of RNase T1 contribute to stabilization of the catalyzed reaction transition state (Loverix and Steyaert, 2001). Their structural counterparts in α-sarcin are Tyr-48, Arg-121 and Leu-145. Site-directed mutagenesis of the two latter residues suggested their involvement in the exquisite specificity of ribotoxin catalysis (Masip et al., 2001, 2003). Here we report the preparation and characterization of a mutant of α-sarcin in which Tyr-48 was substituted by a Phe residue (Y48F). Removal of the -OH group from the phenolic ring abolishes the specific reaction of α-sarcin on rRNA.

Results

Y48F α-sarcin variant production and structural characterization

A mutant of α-sarcin in which the Tyr-48 residue was substituted by Phe (Y48F) was produced in *E. coli* and purified to homogeneity (ca. 3 mg/l of original bacterial culture), according to its electrophoretic behavior in SDS-PAGE (Figure 2A) and amino acid analysis. This purified protein was indeed recognized in immunoblotting using α-sarcin rabbit antiserum (Figure 2B). The calculated $E^{0.1\%}$ (280 nm, l=1 cm) value was 1.21, while that reported for the wild-type protein is 1.34 (Gavilanes et al., 1983), in good agreement with the expected value after removal of one Tyr side chain.

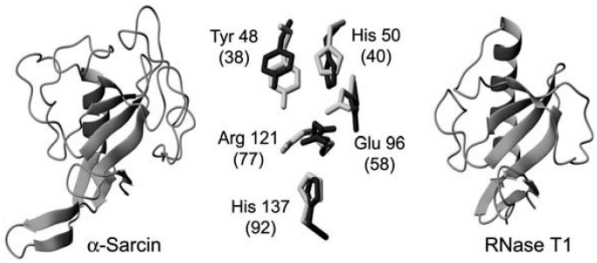

Figure 1 Representation of the structures and active site residues of α-sarcin and RNase T1.
The images were generated with the MOLMOL program (Koradi et al., 1996) from the atomic coordinates deposited in PDB (entries 1DE3 and 1RNT, respectively). The side-chain residues corresponding to the active site of both α-sarcin (black) and RNase T1 (gray) are also represented. Numbers within brackets correspond to RNase T1.

The native conformation of the wild-type protein was fully preserved in the mutant, since the far-UV circular dichroism spectra of both proteins were coincident (Figure 3A). The spectral features in the near-UV range were significantly different, but again in agreement with the removal of a tyrosine side-chain (Figure 3B). Fluorescence emission spectra for both wild-type and Y48F α-sarcin were almost identical (Figure 3C,D), revealing the lack of fluorescence emission of Tyr-48 in the wild-type protein. The thermal denaturation profile for the mutant protein was indistinguishable from that for wild-type α-sarcin (Figure 4), reinforcing the notion that the natural protein fold is conserved in Y48F.

The complexity of the NMR spectrum of globular proteins primarily results from conformation-dependent chemical shift dispersion. This arises because buried peptide groups in globular proteins are shielded from the solvent and are nearest neighbors to other peptide segments, so that different residues experience different structural environments. To obtain a more detailed picture of the conformational arrangement in the Y48F mutant, structural analysis was also performed by NMR spectroscopy. Figure 5 shows the 1D proton spectrum of the Y48F α-sarcin mutant. Assignments for several protons in the mutant could be made by comparison with the wild-type protein (Campos-Olivas et al., 1996; Pérez-Cañadillas et al., 2000) and some of them (Hγ Pro-98, Me Thr-53, Hα Phe-100, Hδ His-150, Hζ Phe-31, NH Gly-72, HN Trp-4, HN Cys-6, HN Phe-71, and HNε Trp-4) are labeled in the Figure. Global inspection of the spectrum confirmed that the Y48F mutation did not produce significant observable changes in the dispersion of the protein signals. As can be observed (Figure 5), large chemical shift dispersion in the HN, Hα and aliphatic regions was preserved in the spectrum of the mutant. For example, the chemical shifts for Hγ Pro-98, which have unusual characteristic upfield values (-0.30 and -0.80 ppm) in wild-type (WT) α-sarcin because of their spatial proximity to the aromatic rings of Tyr-48 and Phe-

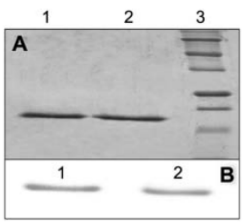

Figure 2 Electrophoretic analysis.
(A) SDS-PAGE of the isolated WT α-sarcin (1) and its Y48F mutant (2); (3) Bio-Rad Low Range Molecular Weight standard marker proteins (phosphorylase b, 97.4 kDa; serum albumin, 66.2 kDa; ovoalbumin, 45 kDa; carbonic anhydrase, 31 kDa; trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa). (B) Western blot analysis of WT (1) and Y48F (2) α-sarcin.

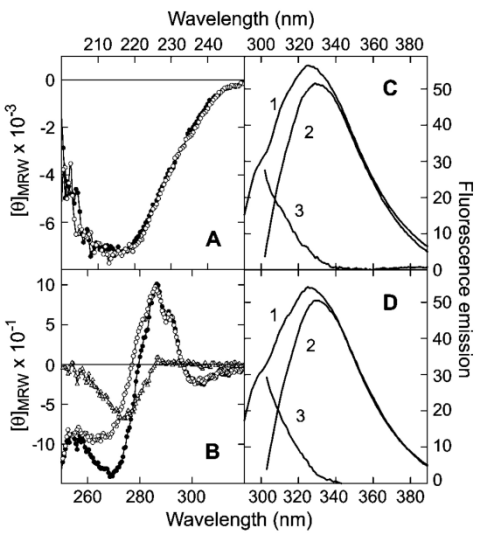

Figure 3 Spectroscopic characterization.
(A) Far- and (B) near-UV circular dichroism spectra of WT α-sarcin (●) and the Y48F mutant (○). Subtraction of the Y48F spectrum from that corresponding to the WT α-sarcin results in the third spectrum (Δ) shown in (B). Mean residue weight ellipticity (θ_{MRW}) is expressed in deg cm^2 dmol^{-1}. (C,D) Fluorescence emission spectra of WT and Y48F proteins, respectively. All spectra were recorded at identical protein concentrations. Spectra labeled '1' resulted from excitation at 275 nm and spectra labeled '2' from excitation at 295 nm (tryptophan contribution). These spectra were normalized at wavelengths above 380 nm. Spectra '3' (tyrosine contribution) were calculated by subtracting spectra '2' from spectra '1'. Fluorescence emission units were arbitrary, and referred to the maximum value of WT α-sarcin.

Table 1 Chemical shift values relative to DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt) of some HN, Hα, Hβ, and aromatic protons of WT α-sarcin and the Y48F mutant at 15°C and pH 5.5.

Residue	δ (ppm)							
	NH		Hα		Hβ		Aromatic	
	WT	Y48F	WT	Y48F	WT	Y48F	WT	Y48F
Ser-47	7.88	7.95	4.30	4.60	4.0	NA	–	–
XX-48	8.80	8.85	4.89	4.86	4.9, 3.1	5.0, 2.4	7.1, 6.8	7.1, 7.3, 7.3
Pro-49	–	–	5.83	5.75	3.1, 2.6	2.9	–	–
His-50	7.89	7.90	5.53	5.40	4.0, 3.7	3.9, 3.6	8.2, 7.2	7.1
Glu-96	8.30	8.41	5.77	5.60	1.8, 1.7	1.8, 1.6	–	–
Phe-97	8.30	8.40	5.17	5.13	3.7, 2.3	3.7, 2.5	8.2, 7.8, 7.3	7.8, 7.2
Phe-100	9.82	9.85	5.85	5.76	3.5, 2.7	3.4, 2.6	7.6, 7.1, 7.1	7.4, 7.1, 7.0
His-137	8.80	8.80	4.91	5.10	3.7, 3.1	3.2	9.0, 7.1	8.9, 7.0

NA, not assigned.

97, were -0.61 and -1.11 ppm in the Y48F mutant. Taking into account that proton chemical shifts are extremely sensitive to ring-current effects and that small variations in the relative orientation of the aromatic ring can cause dramatic changes in the chemical shift values of nearby protons, the small differences found (Δδ 0.3 ppm) were considered good indicators of the preservation of the three-dimensional structure in this mutant. In addition, the similarity between the chemical shift values for HN, Hα, and aromatic protons from different regions of the protein structure also confirm the global structural coincidence between Y48F and the WT protein. Interestingly, the assignments obtained for the active site protons (Table 1) are also in good agreement with those reported for the WT protein, indicating structural conservation of the region where the mutation has taken place. On the other hand, minor conformational changes, due only to the replacement of one side chain (Tyr) by another one of very similar features (Phe), could also be detected by analyzing the pattern of short- and long-range NOE interactions. In this regard, detailed analysis of the spatial interaction was also performed. Some intraresidual (HN-Me Thr-99), short-range (aromatic Trp-51-Me Thr-53; aromatic Phe-100-Hβ, Hγ Pro-98) and long-range (aromatic Phe-108-Me Ala-37, aromatic Tyr-56-Me Leu-62, aromatic Trp-4-Me Ile-134) NOEs were labeled to show significant examples (Figure 5). In all cases, these NOEs involving different types of protons agree with those observed in the WT protein. Importantly, all NOE values for the aromatic protons of Phe-48 were maintained as in the WT protein.

Enzymatic characterization of the Y48F variant

Four different enzyme assays were used to evaluate the ribonucleolytic activity of the Y48F mutant ribotoxin (Kao et al., 2001; Martínez-Ruiz et al., 2001), differing in the substrate employed and thus in the specificity and kinetics of the cleavage. The most specific was that for the natural target of ribotoxins, the ribosome. This activity was measured using a cell-free rabbit reticulocyte lysate. The highly specific action of α-sarcin on a unique phosphodiester bond of 28S RNA renders a 400-nt fragment (α-fragment) that can be visualized after resolving the ribosomal RNA species on an agarose gel (Figure 6A). In decreasing order of complexity, the second substrate used was a 35-mer oligoribonucleotide that mimics the SRL sequence of the ribosome. α-Sarcin specifically cleaves this oligomer, producing two fragments that can be fractionated on polyacrylamide gel (Figure 6B). The third and less specific assay is a zymogram, in which the ribonucleolytic activity is revealed against a homopolymer, poly(A) in this case, embedded in a polyacrylamide gel after electrophoretic separation by SDS-PAGE and convenient renaturation by elimination of the detergent (Figure 6C). The Y48F mutant was inactive against all these three substrates under standard assay conditions (Figure 6), although it completely degraded, without any specificity, the SRL substrate for an incubation time of 16 h, while WT α-sarcin retained its specificity (data not shown). All these results indicate that the Tyr-48 side-chain role was essential for the specific catalytic reaction against rRNA substrate. The fourth type of substrate used was the dinucleoside phosphate ApA, which allows quantification of the kinetic parameters. In this case, only minor differences were observed between Y48F and WT α-sarcin in terms of K_m and k_{cat} (Table 2), revealing that the mutant retains ribonucleolytic activity at this level.

Interaction with DMPG vesicles

Ribotoxins must enter their target cells to gain access to ribosomes and inactivate them. It is well established that this cell-penetrating ability is directly related to the interaction with bilayer membranes rich in acid phospholipids (Gasset et al., 1989, 1994; Martínez-Ruiz et al., 2001).

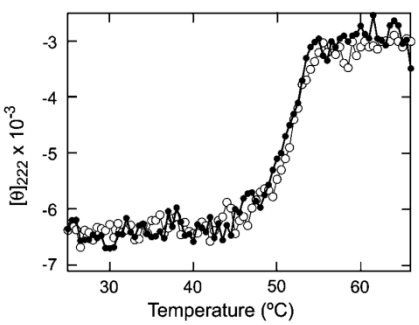

Figure 4 Thermal denaturation profiles for WT α-sarcin (●) and Y48F (○) measured by recording the ellipticity at 222 nm (θ_{222}). θ_{222} is given in units of deg cm^2 dmol^{-1}.

Table 2 Kinetic parameters calculated for the transesterification of ApA by linear regression analysis of double reciprocal plots as described in Lacadena et al. (1998).

Protein	ApA		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
WT[a]	40.0	1.0×10^{-4}	2.5
Y48F	43.0	7.0×10^{-5}	1.63

[a]Previously reported in Lacadena et al. (1998).

Thus, α-sarcin can interact with DMPG-containing liposomes, leading to aggregation and fusion (Gasset et al., 1989) and resulting in the operational translocation of the protein across the bilayer of these model membranes (Oñaderra et al., 1993). On the other hand, it has also been reported that some of the active-site mutants studied so far showed significant modification of this behavior (Masip et al., 2001). These results were interpreted in terms of the possibility that proteins evolved to interact with RNA and thus would have developed structural determinants to recognize polyphosphate lattices that might allow the recognition of a surface phospholipid bilayer (Masip et al., 2001). Within this context, the ability of Y48F α-sarcin to induce the aggregation of DMPG model vesicles was also studied. In this case, the mutant protein promoted identical interaction with the vesicles as the WT fungal ribotoxin (Figure 7).

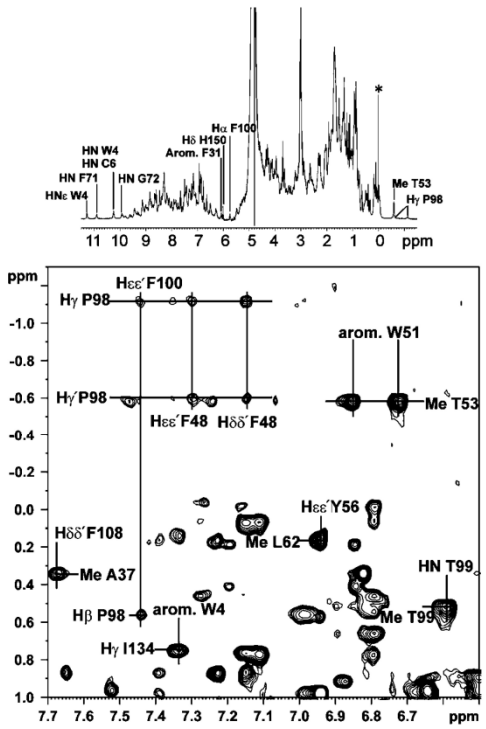

Figure 5 NMR characterization of the Y48F α-sarcin mutant. Top panel: ^{1}H NMR spectrum of Y48F variant. Regions corresponding to the NH and Hα resonances are indicated, together with the assignment of well-isolated signals. The chemical shift reference peak (DSS) is labeled with an asterisk. Bottom panel: selected region of the two-dimensional ^{1}H-^{1}H NOESY spectrum of Y48F showing some intraresidual (HN-Me Thr-99) short-range (aromatic Trp 51-Me Thr-53; aromatic Phe-100-Hβ, Hγ Pro-98) and long-range (aromatic Phe-108-Me Ala-37, aromatic Tyr-56-Me Leu-62, aromatic Trp-4- Me Ile-134) NOEs.

Discussion

The spectroscopic characterization of Y48F α-sarcin strongly suggests that the WT protein fold is conserved. Thus, no differences were observed in the far-UV CD spectrum (Figure 3A). The changes observed in the near-UV range are easily explained in terms of substitution of the phenolic ring of a tyrosine residue by the benzene ring of phenylalanine (Figure 3B). The fluorescence emission spectra only showed very minor differences (Figure 3C,D), also suggesting that the emission of Tyr-48 is highly quenched in the WT protein, as would be expected for a residue side-chain taking part in a complex network of molecular interactions. No differences were detectable in terms of the thermal stability of the mutant in comparison with the WT protein (Figure 4). Overall, these results are even more remarkable if it is taken into account that the CD and fluorescence spectra of WT α-sarcin are largely conditioned by the presence of the aromatic ring of Trp-51 (De Antonio et al., 2000) and that His-50 is one of the conserved residues located within the active site of the protein (Lacadena et al., 1999). The NMR characterization reinforces this conclusion. The ^{1}H NMR spectrum of a globular protein displays large signal dispersion and a number of well-resolved resonances characteristic of the folded conformation, as observed in Figure 5 for the Y48F mutant. Considering the above spectroscopic results and the fact that proton NMR is a particularly sensitive method for testing folded conformations and detecting subtle conformational changes in proteins in aqueous solution, it is then reasonable to assume that only very minor local changes, not detected by ^{1}H NMR, around the Tyr-48 environment could occur upon its replacement by Phe (Figure 5). In addition, the Y48F molecule did not exhibit any difference to the WT protein upon interaction with lipid vesicles. The results shown therefore not only indicate preservation of the

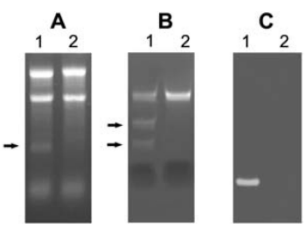

Figure 6 Enzymatic characterization of WT α-sarcin (1) and its Y48F mutant (2).
(A) Cell-free reticulocyte lysate assay (denaturing 2.4% agarose gels). The arrow indicates the 400-nt α-fragment. (B) 35-mer SRL assay (denaturing 19% polyacrylamide gel). Arrows indicate the 21- and 14-mer fragments. (C) Zymogram on a poly(A)-containing gel (15% polyacrylamide, 0.1% SDS).

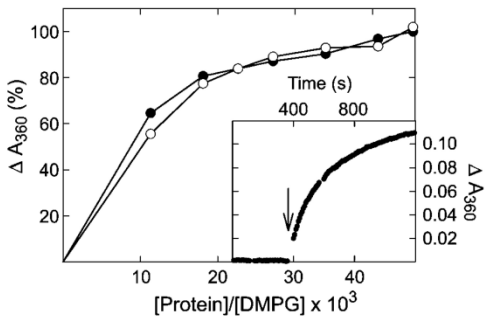

Figure 7 DMPG vesicle aggregation promoted by WT α-sarcin (●) and its Y48F mutant (○) measured by the apparent absorbance increase at 360 nm (ΔA_{360}).
Inset: example of the absorbance increment versus time. The arrow marks the time point for protein addition.

overall fold in the mutant studied, but also suggest that the geometric arrangement of the active-site side-chain residues of the enzyme were scarcely modified.

However, substitution of Tyr-48 by Phe resulted in a mutant lacking ribonucleolytic activity against polymeric RNA substrates, including ribosomes (Figure 6; Table 2), although extensive non-specific degradation of the SRL oligo was observed after long-term incubation. Thus, the Y48F substitution abolished the activity of α-sarcin to specifically release the α-fragment from ribosomes.

As mentioned above, ribotoxins are considered members of a family of fungal secreted RNases represented by RNase T1, a model enzyme based on the extent of studies of its activity-structure relationships. The RNase T1-catalyzed reaction consists of transphosphorylation following an in-line mechanism implying a base and an acid at either side of the scissile bond (Loverix and Steyaert, 2001). Glu-58 and His-92 of RNase T1 are the catalytic base and acid, respectively, and Glu-96 and His-137 are their structural counterparts in α-sarcin. The base and acid catalytic role of these two residues has been demonstrated by preparing α-sarcin mutants at these positions (Lacadena et al., 1999). In the crystal complex of RNase T1 with the minimal substrate 3′-GMP (Loverix and Steyaert, 2001), Tyr-38, His-40, Arg-77 and Phe-100 appear to be part of the catalytic site of the enzyme. His-40 plays a critical role, most probably orientating the nucleophile for attack on the phosphorous. The structurally equivalent His-50 residue of α-sarcin also displays a similar role in α-sarcin catalysis (Lacadena et al., 1999). Regarding the other three residues in the active site of RNase T1, it has been speculated that they form a prearranged structural and dielectric microenvironment that is complementary in shape, charge, and hydrogen bonding capacity to the equatorial oxygens of the transition state, contributing to its optimal solvation/desolvation (Loverix and Steyaert, 2001). Tyr-48, Arg-121 and Leu-145 are the three corresponding structural counterparts in α-sarcin. The side chain of Phe-100 is an apolar catalytic element, stabilizing charge separations that occur in the transition state by controlling the dielectric environment (Doumen et al., 1996). Its structural equivalent in α-sarcin is Leu-145. The L145F variant of α-sarcin is an active ribonuclease (the mutant exhibited similar K_m and slightly lower catalytic efficiency against ApA substrate), but displays lower specificity against rRNA and SRL substrates than the WT protein (Masip et al., 2003). Arg-77 of RNase T1 might facilitate the nucleophilic attack, although it has not been proven by site-directed mutagenesis. Arg-121 is the structural counterpart in α-sarcin. Substitutions of this residue failed to produce degradation of RNA substrates, although the mutants were still active against ApA (similar K_m and lower catalytic efficiency than the WT protein; Masip et al., 2001). Tyr-38 of RNase T1 forms a short hydrogen bond with O2P in the RNase T1/3′-GMP complex and this interaction may improve in the transition state (Loverix and Steyaert, 2001). The equivalent Tyr-48 of α-sarcin only slightly contributes to the catalytic efficiency against ApA, but was revealed as essential for the characteristic ribotoxin activity (specific degradation of rRNA and SRL substrates). Thus, Tyr-48, Arg-121, and Leu-145 appear to be determinants of the ribotoxin activity of α-sarcin.

Studies of the crystal structures of complexes of the α-sarcin-like ribotoxin restrictocin with inhibitors led to the proposal that these ribotoxins may use base flipping to enable cleavage at the correct site of the SRL substrates (Yang et al., 2001). This base flipping may be a common cleavage mechanism for endonucleases acting on folded substrates, as is the case for ribotoxins (Yang et al., 2001). Therefore, Tyr-48 could be part of the machinery that makes ribotoxins one of the most selective examples of specificity, selecting a unique phosphodiester bond among all of the thousands present in the ribosomes. As mentioned above, changing this residue to Phe resulted in an active ribonuclease that in the long term extensively degraded SRL substrates, but failed to produce the expected specific cleavage on either rRNA or SRL-like oligonucleotides.

Previous studies have demonstrated that the NH_2-terminal β-hairpin (positions 7–22) of α-sarcin would direct the ribotoxin to the SRL region of the ribosome (García-Ortega et al., 2002). Three Lys residues of the ribotoxin restrictocin, Lys-110, Lys-111 and Lys 113 (Lys-111, Lys-112 and Lys-114 in loop 3 of α-sarcin), contact the identity element of the ribosomal SRL region (guanine G4319, which forms the bulged G-motif-type S-turn that docks with ribotoxins; Yang et al., 2001). Finally, Tyr-48, Arg-121, and Leu-145 would enable the base flipping performed by His-50/Glu96/His-137 that permits ribonuclease cleavage at a unique phosphodiester bond. Given how little is known about the molecular basis of how ribonucleases for folded substrates select a particular target site, this study represents a further step in shedding more light on the mechanism of action of this family of highly specific apoptotic ribotoxins.

Materials and methods

DNA manipulations

All materials and reagents were of molecular biology grade. Cloning procedures, oligonucleotide site-directed mutagenesis (Kunkel et al., 1987), and bacterial manipulations were carried

out as previously described (Lacadena et al., 1994; Martínez-Ruiz et al., 2001). The mutagenic primer used to substitute Tyr-48 by Phe was 5′-AGC AGC TTT CCT CAC TGG-3′ (the base that changes the Tyr codon to Phe is underlined). Presence of the mutation was confirmed by sequencing the complete cloned cDNA. The plasmid used as the template for mutagenesis experiments, containing the cDNA sequence coding for WT α-sarcin, has already been described (Lacadena et al., 1994, 1999).

Protein production and purification

E. coli BL21 (DE3) cells cotransformed with a thioredoxin-producing plasmid (pT-Trx) and the corresponding α-sarcin mutant plasmids were used to produce the mutant, as previously described (Lacadena et al., 1994, 1999; García-Ortega et al., 2000). Fungal WT α-sarcin was obtained as previously reported (Martínez-Ruiz et al., 2001). Protein purification included ion exchange and molecular exclusion chromatographies (Martínez-Ruiz et al., 2001). PAGE of proteins, protein hydrolysis, and amino acid analysis were performed according to standard procedures (Lacadena et al., 1994).

Spectroscopic characterization

Absorbance measurements were performed on an Uvikon 930 spectrophotometer at room temperature in cells with a 1-cm optical path length at a scanning speed of 100 nm/min. Circular dichroism (CD) spectra were obtained on a Jasco 715 spectropolarimeter, equipped with a thermostated cell holder and a NesLab-111 circulating water bath, at 0.2 nm/s. The instrument was calibrated with (+)-10-camphorsulfonic acid. CD spectra were recorded in cylindrical cells with an optical path length of 0.1 and 1.0 cm. Mean residue weight ellipticity is expressed in units of deg cm^2 $dmol^{-1}$. Thermal denaturation profiles were obtained by measuring the temperature dependence of the ellipticity at 222 nm in the range 25–85°C; the temperature was continuously changed at a rate of 0.5°C/min. Fluorescence emission spectra were recorded on an SLM Aminco 8000 spectrofluorimeter at 25°C using a slit width of 4 nm for both excitation and emission beams. The spectra were recorded for excitation at 275 and 295 nm and both were normalized by considering that Tyr emission above 380 nm is negligible. The Tyr contribution was calculated as the difference between the two normalized spectra. Thermostated cells with a path length of 0.2 and 1.0 cm for the excitation and emission beams, respectively, were used. The temperature was controlled using a circulating water bath. NMR spectra were obtained on a Bruker AMX600 spectrometer at 25°C using standard pulse sequences with pre-saturation or including a Watergate module for elimination of the water signal. ^{1}H homonuclear TOCSY with a mixing time of 60 ms and ^{1}H-^{1}H NOESY spectra with a mixing time of 50 ms were recorded. Samples for NMR experiments were prepared by dissolving the lyophilized protein in H_2O/D_2O (9:1 v/v) at an approximate concentration of 1 mM, and the pH (not corrected for isotope effects) was adjusted to 5.5.

Ribonucleolytic activity

The ribonucleolytic activity of α-sarcin on ribosomes was followed by detecting the release of the 400-nt α-fragment from a cell-free reticulocyte lysate. Production of the 400-nt α-fragment was visualized by ethidium bromide staining after electrophoresis on denaturing 2.4% agarose gels (Figure 6). The specific cleavage by α-sarcin of an SRL-like synthetic 35-mer RNA was also studied. The synthesis of this SRL RNA was carried out as described by Kao et al. (2001). The assay was performed with 2 μM SRL RNA and incubation for 20 min at 37°C in 10 mM Tris-HCl buffer, pH 7.0. The reaction products were detected by ethidium bromide staining after electrophoretic separation on a denaturing 19% (w/v) polyacrylamide gel. The specific action of α-sarcin produced 21- and 14-mer fragments. In addition, the activity of the purified proteins against homopolynucleotides (zymogram) was also assayed. Volumograms of the electrophoretic bands (based on integrating all of the pixel intensities comprising each spot) were obtained with the UVI-Tec photo documentation system and UVIsoft UVI band Windows Application V97.04. These data were used to quantify the enzyme activity in the different assays. In addition, enzymatic hydrolysis of the dinucleoside phosphate at pH 7.0 was also performed as described elsewhere (Lacadena et al., 1998) and the reaction products were fractionated and quantified by HPLC. All these enzyme assays were performed as previously described (Kao et al., 2001). Convenient controls were routinely performed to test potential non-specific degradation of the substrates, which did not occur under the conditions used.

Phospholipid vesicle aggregation

Dimyristoylphosphatidylglycerol (DMPG) was purchased from Avanti Polar Lipids Inc. (Alabaster, USA). Vesicles were formed by hydrating a dry lipid film with 15 mM Tris, pH 7.0 containing 0.1 M NaCl and 1 mM EDTA for 60 min at 37°C. This lipid suspension was then subjected to five cycles of extrusion through two stacked 0.1-μm (pore diameter) polycarbonate membranes (Mancheño et al., 1994). The average diameter of the vesicle population was 100 nm (85% of the vesicles in the range 75–125 nm), as determined by electron microscopy studies (Mancheño et al., 1994). Aggregation was monitored as previously described (Gasset et al., 1989) by measuring the increase in absorbance at 360 nm of a suspension of vesicles (30 μM final lipid concentration) after addition of a small aliquot of a freshly prepared solution of protein (Figure 7).

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A2. Papel del carácter básico de la horquilla β amino-terminal de la α-sarcina en el reconocimiento del ribosoma y en la interacción con fosfolípidos.

Los residuos 1-26 de la α-sarcina forman una gran horquilla β que puede considerarse compuesta por dos horquillas β consecutivas conectadas por una región bisagra. La parte más distal de esta gran horquilla (que comprende la secuencia 7-22) aparece como una protuberancia de la proteína y es una de las regiones con mayor variabilidad de secuencia entre las ribotoxinas. La caracterización previa de una variante de la α-sarcina carente de esta protuberancia mostró que se trata de una región esencial para la citotoxicidad de la proteína. Estudios con modelos de ajuste, enzimáticos y de interacción lípido-proteína sugirieron que la horquilla β amino-terminal está implicada en la interacción con membranas celulares y que establece interacciones específicas con proteínas ribosomales, orientando el centro activo de la α-sarcina hacia el SRL. Con el objetivo de analizar la influencia del carácter básico de esta horquilla β amino-terminal en la actividad citotóxica de las ribotoxinas (pues a lo largo de sus 16 residuos se encuentran 1 arginina y 4 lisinas), se produjeron, purificaron a homogeneidad y caracterizaron cinco mutantes puntuales en los que esos cinco residuos básicos se sustituyeron por ácido glutámico. En cuanto al reconocimiento del ribosoma, todos los mutantes mostraron una menor actividad que la proteína silvestre frente a un lisado de reticulocitos libre de células, mientras que la actividad frente a un oligorribonucleótido sintético que mimetiza el SRL o frente al homopolímero poli(A) no se vio afectada, confirmando que los residuos básicos mutados participan en interacciones electrostáticas con otros elementos ribosomales distintos del SRL y que estas interacciones mejoran el reconocimiento entre la enzima y su sustrato. El estudio de la interacción con vesículas de fosfolípidos mostró que la Lys 17, la Arg 22 y, sobre todo, las Lys 14 y 21 son residuos cruciales en las primeras etapas del fenómeno de agregación, en el que se requieren interacciones proteína-vesícula y proteína-proteína. En resumen, los datos obtenidos revelan que las interacciones electrostáticas en las que participan los residuos básicos de la horquilla β son esenciales no sólo para establecer interacciones específicas con regiones ribosomales distintas al SRL sino también para explicar la capacidad de la proteína para interaccionar con bicapas de fosfolípidos ácidos.

Trabajo A2: **Álvarez-García E**, Martínez del Pozo Á y Gavilanes JG (2009a) Role of the basic character of α-sarcin's NH_2-terminal β-hairpin in ribosome recognition and phospholipid interaction. Arch Biochem Biophys. 481: 37-44.

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Role of the basic character of α-sarcin's NH_2-terminal β-hairpin in ribosome recognition and phospholipid interaction

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Phospholipid vesicles aggregation

ABSTRACT

Ribotoxins are a family of toxic extracellular fungal RNases that first enter into the cells and then exert a highly specific ribonucleolytic activity on the larger rRNA molecule, leading to protein synthesis inhibition and cell death by apoptosis. α-Sarcin is the best characterized ribotoxin. Previous characterization of a deletion variant of this protein showed that its long NH_2-terminal β-hairpin is essential for its cytotoxicity. Docking, enzymatic, and lipid–protein interaction studies suggested that this β-hairpin establishes specific interactions with ribosomal proteins and that it is a region involved in the interaction with cell membranes. Consequently, in order to assess the influence of the basic character of this NH_2-terminal β-hairpin (there are 1 arginine and 4 lysines along its 16 residues) on the ribotoxins cytotoxic ability, five individual mutants substituting these five basic residues by glutamic acid were produced, purified to homogeneity, and characterized. Regarding ribosomal recognition, all mutants showed a diminished activity in a cell-free reticulocyte lysate, whereas the activity against an oligoribonucleotide mimicking the sarcin/ricin loop rRNA (SRL) or the homopolymer poly(A) remained unaffected, confirming that the mutated basic residues participate in electrostatic interactions with other ribosomal elements apart from this SRL. The study of the interaction with phospholipid vesicles showed that Lys 17, Arg 22, and, most importantly, Lys 14 and Lys 21, are crucial residues in the first stages of the aggregation phenomenon, where protein–vesicle and protein–protein interactions are required. The data obtained reveal that electrostatic interactions involving basic residues of the β-hairpin are required not only for establishing specific interactions with ribosomal regions other than the SRL but also to explain the ability of the protein to interact with acid phospholipid bilayers.

Introduction

α-Sarcin is the most representative member of ribotoxins, a family of fungal natural killers characterized by their exquisite ribonucleolytic specificity against ribosomes and their ability to cross cellular membranes in the absence of any known protein receptor [1,2]. These toxic proteins cleave just a single phosphodiester bond of the large rRNA fragment, located at an evolutionarily conserved loop with important roles in ribosome function [3–5]. This cleavage inhibits protein biosynthesis, leading to cell death by apoptosis [6]. This important region has become to be known as the sarcin/ricin loop (SRL)[1] because it is not only the target of α-sarcin and the rest of ribotoxins but also of the much larger group of plant ribosome-inactivating proteins (RIP), best represented by ricin [7,8].

Most ribotoxins show a high degree of sequence identity [9–12] that is also manifested in the three-dimensional structure of the two only ribotoxins studied at this level, restrictocin [13,14] and α-sarcin [15–18]. Both proteins fold into an α+β structure with a central five-stranded antiparallel β-sheet and an α-helix of almost three turns and display long and unstructured loops (Fig. 1) [15,19,20]. Residues 1–26 form a long NH_2-terminal β-hairpin that can be considered as two consecutive minor β-hairpins connected by a hinge region with its most distal part jutting out as a solvent exposed protuberance (Fig. 1). This β-hairpin is one of the regions showing the highest sequence variability among ribotoxins [9–12,21]. An α-sarcin mutant involving the deletion of this protuberance, α-sarcin Δ(7–22), retained the same conformation as the wild-type protein, as ascertained from its three-dimensional structure in solution [22]. However, functional and enzymatic studies revealed that this mutant exhibited ribonuclease activity against naked rRNA and synthetic substrates but lacked the ability to specifically cleave the SRL in intact ribosomes [23]. These results were explained by *in silico* studies that predicted how this NH_2-terminal β-hairpin could

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[1] *Abbreviations used:* SRL, sarcin/ricin loop; RIP, ribosome-inactivating proteins; CD, circular dichroism; DMPG, dimyristoylphosphatidylglycerol.

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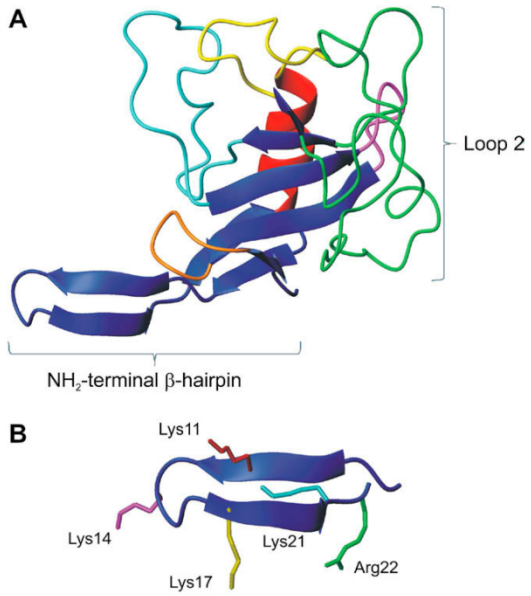

Fig. 1. Diagrams showing the three dimensional structure of α-sarcin (A) and a detail of its NH_2-terminal β-hairpin (B). The different basic residues mutated to Glu are shown.

establish essential interactions, mostly of electrostatic nature, with specific ribosomal proteins in order to direct the ribotoxin to the SRL region of the ribosome [18].

In addition to their specific and lethal ribonucleolytic activity, ribotoxins can also cross phospholipid membranes due to their ability to interact with acid phospholipid-containing bilayers [6,24–28]. This is the basis to explain why they are especially active on transformed or virus-infected cells [6,29,30], although any ribosome could be potentially inactivated by them, given the universal conservativeness of the SRL. According to the current model accepted to explain the ability of α-sarcin to interact with phospholipid bilayers, the protein would be initially adsorbed to the charged polar head groups of the phospholipids, and then would partially penetrate the interface of the bilayer to interact with a portion of the lipid hydrocarbon chains [1,6,26,31,32]. This intercalation within the lipid matrix would promote fusion and permeability changes in the bilayers, processes that would presumably be involved in the passage of the protein across the membrane of its target cells. Two regions of the protein, located at opposite ends of the protein molecule, have been proposed to be specifically involved in vesicle aggregation [33]. These regions would be loop 2 and, again, the NH_2-terminal β-hairpin (Fig. 1), which consequently would also participate in the interaction with cell membranes. In good accordance with this proposal, the α-sarcin Δ(7–22) mutant also displayed a diminished ability to interact with phospholipid lipid vesicles showing a behavior compatible with the absence of one vesicle-interacting region [23]. In agreement with all these conclusions, the deletion mutant exhibited a very low cytotoxicity on human rhabdomyosarcoma cells [23].

α-Sarcin is a highly charged protein, with a high isoelectric point [34,35]. This high content of positively charged residues, mostly located at the loops and the NH_2-terminal β-hairpin, is probably required for recognizing and binding not only to its highly negatively charged target, the SRL rRNA, but also to ribosomal proteins and cellular membranes [18,36,37]. In this context, the role of the positively charged residues located at the NH_2-terminal β-hairpin (Table 1) in these different but closely related events has been studied in the work herein presented.

Table 1
Mutagenic primers used to construct individual mutant versions of wild-type α-sarcin where the NH_2-terminal β-hairpin basic residues had been substituted by Asp. The bases that change original codon to Asp are underlined.

α-Sarcin mutant	Oligonucleotide sequence
K11E	5′ ttg aac gac cag gag aac ccc aag acc 3′
K14E	5′ cag aag aac ccc gag acc aac aag tat 3′
K17E	5′ ccc aag acc aac gag tat gag acc aaa 3′
K21E	5′ aag tat gag acc gaa cgc ctc ctc tac 3′
R22E	5′ tat gag acc aaa gag ctc ctc tac aac 3′

Materials and methods

DNA manipulations

All materials and reagents were of molecular biology grade. Cloning procedures, oligonucleotide site-directed mutagenesis, and bacterial manipulations were carried out as previously described [28,38,39]. The mutagenic primers used to substitute the mutated residues are shown in Table 1. Presence of only the mutation expected in each case was confirmed by sequencing the complete cloned cDNA. The plasmid used as the template for mutagenesis experiments, containing the cDNA sequence coding for wild-type α-sarcin, has already been described [38,40].

Protein production and purification

Escherichia coli BL21 (DE3) cells cotransformed with a thioredoxin-producing plasmid (pT-Trx) and the corresponding α-sarcin mutant plasmids were used to produce the different proteins studied, as previously described [38,40,41]. Fungal wild-type α-sarcin was obtained as previously reported [28]. Protein purification included ion exchange and molecular exclusion chromatographies [28]. PAGE of proteins, Western blot immunodetection, protein hydrolysis, and amino acid analysis were performed according to standard procedures [38,41].

Spectroscopic characterization

Absorbance measurements were performed on an Beckman DU640 spectrophotometer at room temperature in cells with a 1 cm optical path length at a scanning speed of 240 nm/min. Circular dichroism (CD) spectra were obtained on a Jasco 715 spectropolarimeter, equipped with a thermostated cell holder and a NesLab-111 circulating water bath, at 0.2 nm/s. The instrument was calibrated with (+)-10-camphorsulfonic acid. CD spectra were recorded in cylindrical cells with an optical path length of 0.1 and 1.0 cm. Mean residue weight ellipticity is expressed in units of deg cm^2 $dmol^{-1}$. Thermal denaturation profiles were obtained by measuring the temperature dependence of the ellipticity at 220 nm in the range 25–80 °C; the temperature was continuously changed at a rate of 0.5 °C/min. Fluorescence emission spectra were recorded on an SLM Aminco 8000 spectrofluorimeter at 25 °C using a slit width of 4 nm for both excitation and emission beams. The spectra were recorded for excitation at 275 and 295 nm and both were normalized by considering that Tyr emission above 380 nm is negligible. The Tyr contribution was calculated as the difference between the two normalized spectra. Thermostated cells with a path length of 0.2 and 1.0 cm for the excitation and emission beams, respectively, were used. The temperature was controlled using a circulating water bath. All these experiments were made with the proteins

dissolved in 50 mM sodium phosphate, pH 7.0, containing 0.1 M NaCl following procedures described before [28,38].

Ribonucleolytic activity

The ribonucleolytic activity of α-sarcin on eukaryotic ribosomes was followed by detecting the release of the near 400-nts α-fragment from a rabbit cell-free reticulocyte lysate. Production of this fragment was visualized by ethidium bromide staining after electrophoresis on denaturing 2.4% agarose gels. The specific cleavage by α-sarcin of an SRL-like synthetic 35-mer RNA was also studied. The synthesis of this SRL RNA was carried out as described [42]. The assay was performed with 4 μM SRL RNA and incubation for 15 min at 37 °C in 50 mM Tris–HCl buffer, pH 7.0, containing 0.1 M NaCl and 5 mM EDTA. The reaction products were detected by ethidium bromide staining after electrophoretic separation on a denaturing 19% (w/v) polyacrylamide gel. The specific action of α-sarcin produced 21- and 14-mer fragments. In addition, the activity of the purified proteins against homopolynucleotides (zymogram) was also assayed. Volumograms of the electrophoretic bands (based on integrating all of the pixel intensities comprising each spot) were obtained with the UVI-Tec photo documentation system and UVIsoft UVI band Windows Application V97.04. These data were used to quantify the enzyme activity in the different assays. All these enzymatic assays were performed as previously described [28,38,42]. Convenient controls were routinely performed to test potential non-specific degradation of the substrates, which did not occur under the conditions used.

Phospholipid vesicle aggregation

Dimyristoylphosphatidylglycerol (DMPG) was purchased from Avanti Polar Lipids Inc. (Alabaster, USA). Vesicles were formed by hydrating a dry lipid film with 15 mM Tris, pH 7.0 containing 0.1 M NaCl and 1 mM EDTA for 60 min at 37 °C. This lipid suspension was then subjected to five cycles of extrusion through two stacked 0.1-mm (pore diameter) polycarbonate membranes [33]. The average diameter of the vesicle population was 100 nm (85% of the vesicles in the range 75–125 nm), as determined by electron microscopy studies [33]. Aggregation was monitored as previously described [24,28] by measuring the increase in absorbance at 400 nm of a suspension of vesicles (30 mM final lipid concentration) after addition of a small aliquot of a freshly prepared solution of protein.

Results

Protein purification and structural characterization

All six proteins, wild-type and the five single mutants, were purified to homogeneity and in milligram amounts (Table 2), according to their SDS–PAGE behavior (Fig. 2A) and amino acid composition. This composition was consistent with the mutations expected in each case. All them were also detected by a rabbit anti-α-sarcin serum in Western blot assays (Fig. 2B). The amino acid analysis and the corresponding UV-absorbance spectra were used to calculate their extinction coefficients (Table 2).

Table 2
Purification yields (mg per liter of original broth) and spectroscopic properties of the different proteins studied.

Protein	Yield	$E^{0.1\%}$ (280 nm, 1 cm)	Q_{Tyr}[a]	Q_{Trp}[a]
WT	7.0 mg/l[b]	1.34	1.00	1.00
K11E	10.5 mg/l	1.31	1.08	1.09
K14E	17.0 mg/l	1.36	0.92	1.03
K17E	12.5 mg/l	1.36	1.06	1.03
K21E	12.5 mg/l	1.35	1.03	1.06
R22E	12.0 mg/l	1.20	1.03	1.13

[a] Relative quantum yield of Tyr and Trp referred to the values of the wild-type protein.

[b] [41].

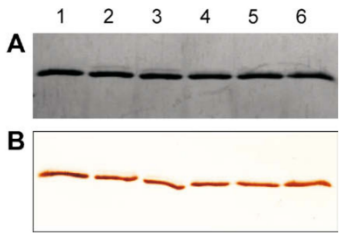

Fig. 2. Analysis by SDS–PAGE of wild-type (1), K11E (2), K14E (3), K17E (4), K21E (5), and R22E (6) versions of α-sarcin. (A) Coomassie Brilliant Blue staining (0.5 μg of protein/lane). (B) Western blot analysis using an anti-(α-sarcin) polyclonal antibody (0.1 μg of protein/lane).

The native conformation of the wild-type protein was fully preserved in all the mutants, according to the coincidence of their far-UV circular dichroism spectra (Fig. 3A). The spectral features in the near-UV range (Fig. 3B) were also very similar with just the mutants showing small differences practically within the error range of this type of determination. Accordingly, fluorescence emission spectra for both wild-type and the

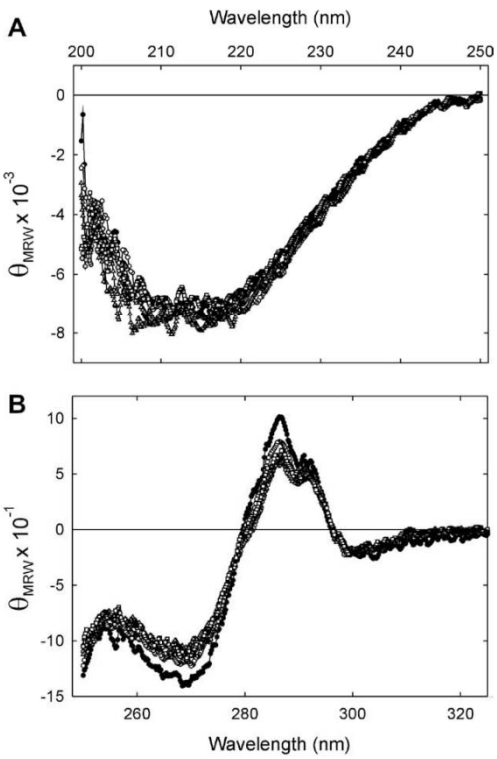

Fig. 3. Circular dichroism spectra in the far (A) and near (B) UV regions, of wild-type (●), K11E (○), K14E (◒), K17E (Δ), K21E (△), and R22E (□) versions of α-sarcin. Mean residue weight ellipticity, θ_{MRW}, is expressed in units of degrees × cm^2 × $dmol^{-1}$.

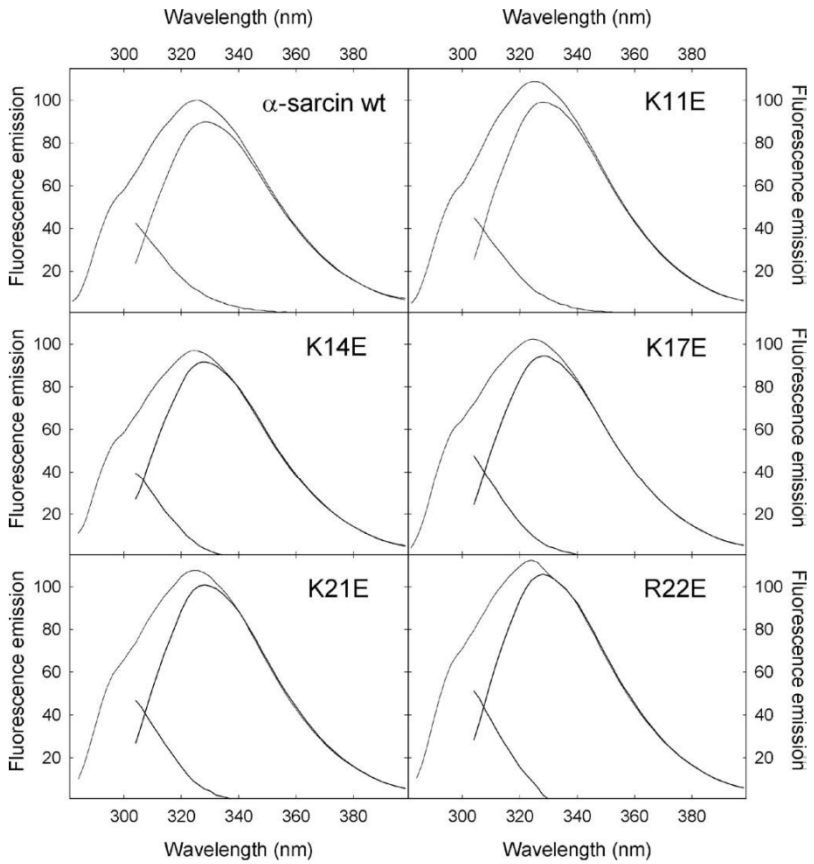

Fig. 4. Fluorescence emission spectra of wild-type, K11E, K14E, K17E, K21E, and R22E versions of α-sarcin at 0.1 mg/ml protein concentration. Spectra 1 were obtained for excitation at 275 nm. Spectra 2 (tryptophan contribution) were obtained for excitation at 295 nm and normalized at wavelengths above 380 nm. Spectra 3 (tyrosine contribution) were calculated as the difference spectra (spectrum 1–spectrum 2). Fluorescence emission is expressed as percentage considering the intensity at the wavelength of the emission maximum of the wild-type protein, for excitation at 275 nm, as 100. All of the spectra were recorded at 25 °C and pH 7.0.

five mutant proteins were also very similar (Fig. 4 and Table 2). These results again suggested that the wild-type protein conformation was retained in the mutants.

All mutants studied showed a slightly decreased conformational stability in comparison with the wild-type protein (Fig. 5) according to their T_m values and the corresponding estimated $\Delta(\Delta G)$ (Table 3). Apparently, substitution of Lys 21 rendered the most unstable mutant. However, the obtained low $\Delta(\Delta G)$ values and the shape of the thermal denaturation profiles (Fig. 5) were also in agreement with the assumption that the mutant proteins displayed an overall conformation very similar to that of wild-type α-sarcin.

Enzymatic characterization

All mutants studied displayed a slightly lower specific ribonucleolytic activity than wild-type α-sarcin when assayed against intact ribosomes in a rabbit reticulocyte lysate (Fig. 6). However, all them behaved indistinguishable when assayed against less specific substrates such as an SRL-like oligonucleotide (Fig. 7) or the hompolynucleotide poly(A) (Fig. 8). Unexpectedly, the α-sarcin K14E mutant was also able to cleave poly(C), an homopolynucleotide resistant to the action of the wild-type protein or the other mutants here studied (Fig. 8).

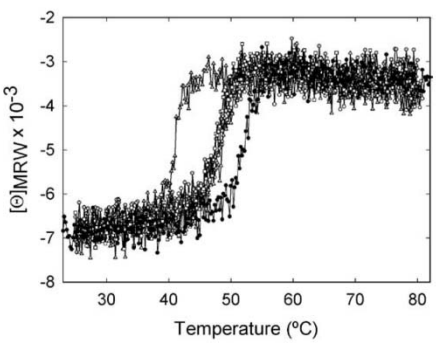

Fig. 5. Thermal denaturation profiles of wild-type (●), K11E (○), K14E (●), K17E (Δ), K21E (Δ), and R22E (□) versions of α-sarcin. The measurements were performed by continuously recording the mean residue weight ellipticity at 220 nm (θ_{MRW}) expressed in units of degrees × cm^2 × dmol^{-1}.

Table 3
Thermodynamic parameters of the different mutants studied.

Protein	T_m (°C)	$\Delta(\Delta G)$ (kcal/mol)[a]
WT	52.0	0.00
K11E	47.0	−2.09
K14E	48.1	−1.62
K17E	48.1	−1.62
K21E	40.9	−4.64
R22E	47.3	−1.97

[a] $\Delta(\Delta G) = (\Delta H \times \Delta T_m/T_m)$ is the stability change produced by the mutation [ΔH, enthalpy change for the wild-type protein (136 kcal/mol) determined at pH 7.0 from differential scanning calorimetric measurements [52]; $\Delta T_m = T_m$ (mutant) − T_m (wild-type); T_m, value obtained for the mutant variant] [53].

Interaction with phospholipid vesicles

In the present study the aggregation of DMPG vesicles promoted by the wild-type protein and the NH_2-terminal β-hairpin mutants was followed by recording the increase of the apparent absorbance at 400 nm of a suspension of DMPG vesicles after addition of a small aliquot of a freshly prepared protein (Fig. 9). The kinetic profiles of this phospholipid vesicles aggregation showed a biphasic behavior (Fig. 9) that was only observed at very low protein/lipid molar ratios for the wild-type α-sarcin and the K11E mutant, as expected from previous studies [33]. On the other hand, the other four mutants studied displayed a much different kinetic pattern of vesicle aggregation being the biphasic pattern much more evident, even at high protein/lipid molar ratios (Fig. 9). These differences were especially significant for mutants K14E and K21E. Apparently, the first aggregation step would be the one being more affected by the mutations, as revealed by the net apparent absorbance change observed at shorter times (Fig. 10). Simultaneously, a delay in the onset of the formation of the structures appearing at longer times was also observed for mutants K14E, K21E, and R22E (Fig. 9), being again much more pronounced at the lower protein concentrations used.

Discussion

The SRL sequence and conformation is maintained in all ribosomes known so far. Furthermore, structural characterization of different SRL-like oligoribonucleotides has revealed how these isolated structures adopt the same conformation as well [43–45], being indeed susceptible of specific recognition and cleavage by ribotoxins [42,46]. The toxin and SRL structural determinants involved in their mutual recognition have been identified and characterized [13–15,47]. However, this cleavage reaction against an isolated SRL-like RNA takes place at rates about 1000-fold slower than when using intact ribosomes [37,46] indicating that additional recognition elements are needed for the optimal ribotoxins' inactivation action against ribosomes. The involvement of the ribosomal context in terms of electrostatic interactions has been suggested not only to justify this recognition and binding enhance-

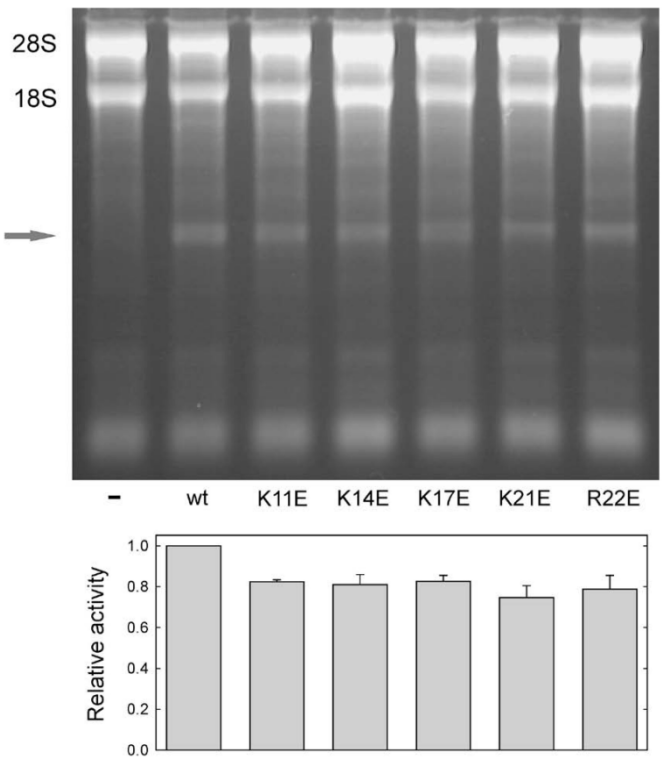

Fig. 6. Ribosome-inactivating activity assay of wild-type, K11E, K14E, K17E, K21E, and R22E versions of α-sarcin. A control in the absence of enzyme is also shown (—). The highly specific ribonucleolytic activity of the ribotoxins is shown by the release of the 400-nt α-fragment (arrow) from the 28S rRNA of eukaryotic ribosomes. Bands corresponding to 28S and 18S RNA are also indicated. Cell-free reticulocyte lysates were incubated in the presence of 100 ng of each protein. The reaction mixture was analyzed on 2.4% agarose gels and stained with ethidium bromide. Quantitation of these activities is also shown (lower panel), taking as unit the activity found for the wild-type protein in these conditions.

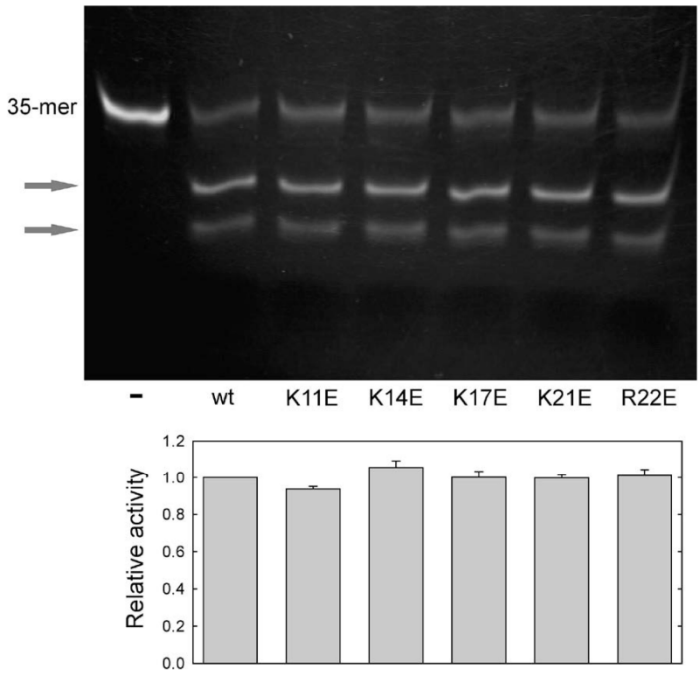

Fig. 7. Activity assay on the 35mer oligonucleotide mimicking the SRL. This substrate was incubated in presence of wild-type, K11E, K14E, K17E, K21E, and R22E versions of α-sarcin (upper panel). A control in the absence of enzyme is also shown (—). Two arrows indicate the position of the 21mer and 14mer oligonucleotides resulting from the specific cleavage of a single phosphodiester bond. The intact 35-mer oligo is also shown. Quantitation of these activities is also shown (lower panel), taking as unit the activity found for the wild-type protein in these conditions.

ment but also to explain the differences found when these toxins are assayed against ribosomes of different origins [18,37]. Electrostatic interactions involving the α-sarcin NH_2-terminal-β-hairpin have been also suggested to mediate α-sarcin specific recognition of ribosomal proteins [18]. In fact, mutation of some basic residues that lie outside the restrictocin-SRL interface has been shown to decrease markedly the ribosomes cleaving rates without having any effect on the kinetics of assays employing an isolated SRL-like oligoribonucleotide [37]. It is also well proven how electrostatic interactions are needed for the establishment of the associations needed to allow the passage of ribotoxins across acid phospholipid membranes [24,25,27] and the α-sarcin NH_2-terminal-β-hairpin is one of the protein regions that has been involved in this membrane recognition mechanism [23,48].

Ribotoxins are basic proteins with high pI values [1] due to their high content of basic residues mostly located at their unstructured loops [13–15]. Five of them concentrate at the α-sarcin NH_2-terminal-β-hairpin being indeed residues 14, 17, 21, and 22 conserved among the different ribotoxins known, with the exception of hirsutellin A, a much smaller ribotoxin of recent characterization [12]. Therefore, with the aim of defining the role of electrostatic interactions involving α-sarcin NH_2-terminal-β-hairpin, these five basic amino acid residues were mutated to Glu and their involvement in the molecular mechanism of α-sarcin cytotoxicity was studied.

Structural characterization of the isolated purified proteins revealed that all retained the wild-type conformation, as indicated by their spectroscopic and thermodynamical characterization. Only minor changes were observed after inspection of the near-UV CD (Fig. 3) and fluorescence emission spectra (Fig. 4), most probably due to the presence of Tyr 18 and 25 and Trp-4, a residue that dominates α-sarcin emission [49], within the sequence of the mutated NH_2-terminal-β-hairpin. All mutants were slightly less stable than the wild-type protein (Table 3) but the differences were even smaller than those ones found among the different natural ribotoxins known [12,48], in good agreement with previous results indicating that the NH_2-terminal-β-hairpin folds as a rather independent structure with low influence on the global thermostability of the protein [23]. This observation is in good accordance with the fact that most of the residues mutated do not establish obvious interactions with any other amino acid of the protein globular core with perhaps the only exception of the non-conserved Lys 11 [15]. The highest difference was found for α-sarcin K21E, being the mutated Lys a residue involved in an electrostatic interaction with Glu-19 [50], an interaction which is not possible for the mutant protein.

Regarding their enzymatic characterization, it seems clear from the results presented that any charge reversion at the NH_2-terminal-β-hairpin results in a diminished specific activity against intact ribosomes (Fig. 6) without affecting the less specific ribo-

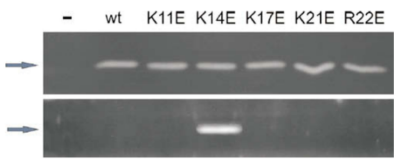

Fig. 8. Zymogram assay of the ribonucleolytic activity against poly(A) and poly(C) (upper and lower panels, respectively). These assays were made at pH 7.0 employing 0.75 μg of wild-type, K11E, K14E, K17E, K21E, or R22E versions of α-sarcin. A control in the absence of enzyme is also shown (—).

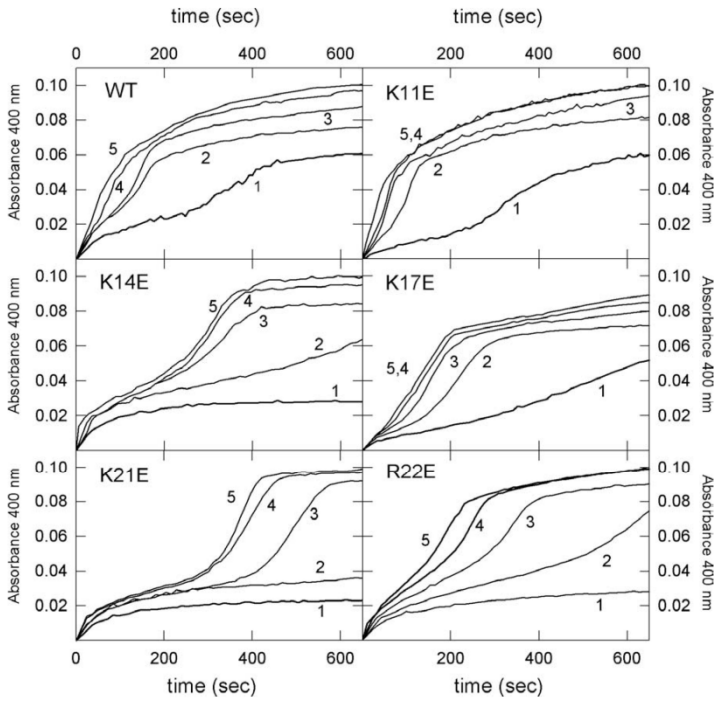

Fig. 9. Kinetic measurement of the effect of the different α-sarcin versions studied on phospholipid vesicle aggregation. The phospholipid/protein molar ratios employed were: 333 (trace 1), 167 (trace 2), 111 (trace 3), 83 (trace 4), and 67 (trace 6). The concentration of DMPG used in these assays was 100 μM.

nucleolytic action of the protein (Fig. 7 and 8). Lys 11 and 14 had been predicted before to establish electrostatic interactions with specific negative residues from ribosomal proteins [18]. The present results would not only confirm this prediction but also suggest that additional interactions are established between the positively charged side-chains at the NH_2-terminal-β-hairpin and negatively charged ribosomal elements, not only proteins but most probably also rRNA included. The observation of the different specificity displayed by mutant K14E against homopolynucleotides (Fig. 8) reveals that the β-hairpin is also involved in their recognition. This involvement might be in terms of a direct interaction with the polymer or most probably due to a modification of the active site accessibility, as has been also suggested before [15,51]. In fact, these solvent accessibility changes were detected before upon deletion of the β-hairpin [22].

It is well known how α-sarcin interacts with lipid vesicles through electrostatic and hydrophobic interactions promoting events of vesicle aggregation that are followed by lipid mixing occurring between the bilayers of the aggregated vesicles, as would be expected for fusing liposomes [1,24,25,28]. Stopped-flow measurement of the initial rates of this aggregation induced by α-sarcin showed a second-order dependence on phospholipid concentration, suggesting the formation of vesicle dimers as the initial steps of the process [33]. These same experiments revealed that protein–protein interactions were involved in the establishment of the needed contacts to maintain the mentioned vesicle dimers [33]. In the present experiments the use of smaller protein concentrations revealed the existence of a biphasic behavior even at the much longer time scale of minutes used now (Fig. 9). Thus, the first step observed would correspond to the formation of small vesicles aggregates, mostly stabilized by protein–protein and protein–vesicle interactions, which would later evolve during the second step of the process to much larger structures involving not only aggregation but also fusion events [24,25,33]. This mechanism would be fully compatible with the biphasic behavior observed in these experiments performed at lower protein/lipid molar ratios (Fig. 9) confirming the current model to explain the ability of ribotoxins to cross phospholipid membranes. Furthermore, the mutant showing more similar behavior to the wild-type protein was K11E, a protein where the mutated residue is not conserved among all ribotoxins, suggesting its minor contribution to the passage of the protein across the membranes. On the other hand, all the other four mutants studied showed a delay affecting the onset of the formation of small vesicle aggregates indicating their participation in maintaining the protein–protein and protein–vesicle interactions needed to initiate the aggregation process. The absence of a single positive charge seems to affect dramatically the accepted mechanism being this effect stronger for replacement of Lys residues 14 and 21. In fact, mutation of these two residues even impairs the formation of the larger aggregates according to the delay also observed for the second stage of the aggregation process. A similar behavior for the myelin basic protein has been attributed to the instability of the protein–protein interactions needed to aggregate the vesicles and most probably this would also be the case for these mutants. Overall, the lipid–protein interaction characterization suggest that residues Lys 14, Lys 17, Lys 21, and Arg 22 do participate directly in the first stages of the vesicle aggregation induced by α-sarcin being much more determinant the role played by residues 14 and 21.

In summary, the result presented show that electrostatic interactions established by the conserved positively charged residues of the α-sarcin NH_2-terminal-β-hairpin are essential not only for

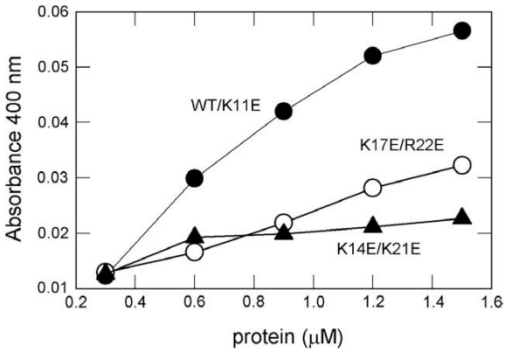

Fig. 10. The extent of phospholipid vesicles aggregation after 100s, corresponding to the first step of the process, is represented against the concentration employed of the different α-sarcin variants studied. The concentration of DMPG used in these assays was 100 μM.

the correct specific recognition of the ribosome but also for the optimal establishment of the phospholipid interactions needed to cross the membranes of its presumed target cells.

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A3. Influencia de algunos residuos clave en la producción extracelular heteróloga de la ribonucleasa U2 en la levadura *Pichia pastoris*.

La ribonucleasa U2, secretada por el hongo *Ustilago sphaerogena*, es una RNasa ciclante con una especificidad bastante inusual dentro del grupo de las RNasas extracelulares microbianas, familia representada por la RNasa T1. La superposición de las estructuras tridimensionales de ambas proteínas sugiere que el residuo equivalente a la histidina 92, esencial en el centro activo de la RNasa T1, es la histidina 101 en la RNasa U2. Por otra parte, la RNasa U2 contiene tres puentes disulfuro, que se establecen entre las cisteínas 1-54, 55-96 y 9-113. La presencia de dos cisteínas consecutivas en la secuencia de la proteína (Cys 54 y Cys 55) podría dar lugar a una formación incorrecta de los puentes disulfuro que quizás constituya la explicación a la existencia de una fracción de proteína mal plegada que aparece en la producción heteróloga de RNasa U2 en *Pichia pastoris*.

Con el objetivo de estudiar ambas hipótesis, se produjeron dos mutantes: U2 H101Q y U2 C1/54S. Su purificación y caracterización desvelaron que la histidina 101 es un residuo esencial para el correcto plegamiento de la proteína, al menos cuando se expresa en la levadura *P. pastoris*. La obtención del mutante U2 H101Q únicamente bajo la forma de una proteína desnaturalizada imposibilitó, sin embargo, la extracción de conclusiones sobre la función puramente catalítica de ese residuo. En cuanto al mutante de las cisteínas, se pudo comprobar que la eliminación del puente disulfuro entre los residuos 1 y 54, el único no conservado de los tres que aparecen en la proteína, no afecta en absoluto al correcto plegamiento de la RNasa U2, aunque se obtenga un mutante mucho menos estable que la proteína silvestre.

Trabajo A3: **Álvarez-García E**, García-Ortega L, de los Ríos V, Gavilanes JG y Martínez del Pozo Á (2009b) Influence of key residues on the heterologous extracellular production of fungal ribonuclease U2 in the yeast *Pichia pastoris*. Protein Expr. Purif. (En prensa)

Influence of key residues on the heterologous extracellular production of fungal ribonuclease U2 in the yeast *Pichia pastoris*.

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Short title: Key residues in the production of ribonuclease U2

Abstract

Ribonuclease U2, secreted by the smut fungus *Ustilago sphaerogena*, is a cyclizing ribonuclease that displays a rather unusual specificity within the group of microbial extracellular RNases, best represented by RNase T1. Superposition of the three-dimensional structures of RNases T1 and U2 suggests that the RNase U2 His 101 would be the residue equivalent to the RNase T1 catalytically essential His 92. RNase U2 contains three disulfide bridges but only two of them are conserved among the family of fungal extracellular RNases. The non-conserved disulfide bond is established between Cys residues 1 and 54. Mispairing of the disulfide network due to the presence of two consecutive Cys residues (54 and 55) has been invoked to explain the presence of wrongly folded RNase U2 species when produced in *P. pastoris*. In order to study both hypotheses, the RNase U2 H101Q and C1/54S variants have been produced, purified, and characterized. The results obtained support the major conclusion that His 101 is required for proper protein folding when secreted by the yeast *P. pastoris*. On the other hand, substitution of the first Cys residue for Ser results in a mutant version which is more efficiently processed in terms of a more complete removal of the yeast α-factor signal peptide. In addition, it has been shown that elimination of the Cys 1-Cys 54 disulfide bridge does not interfere with RNase U2 proper folding, generating a natively folded but much less stable protein.

Keywords: microbial ribonucleases, RNase T1, fungal ribonucleases, disulfide.

Introduction

Ribonuclease U2 (RNase U2) is a single polypeptide chain enzyme of 114 amino acid residues secreted by the smut fungus *Ustilago sphaerogena* [1-4]. This cyclizing ribonuclease displays a rather unusual specificity within the group of microbial extracellular RNases, best represented by RNase T1 [5-7], showing a strong preference for 3'-linked purine phosphodiester linkages [8]. This unique specificity converts RNase U2 in a very useful biotechnological tool of increasing importance regarding RNA sequencing and processing [9-11]. Indeed, this enzyme is the fungal non-toxic RNase which appears to be the phylogenetically closest relative of ribotoxins, a family of highly specific ribosome-inactivating fungal proteins with antitumoral properties [12]. Therefore, RNase U2 is the best model to compare the mechanisms of both toxic and non-toxic groups of microbial RNases. Nevertheless, all these extracellular fungal RNases of the RNase T1 family, ribotoxins included, share an identical structural core [12-15] and comparison of their three-dimensional structures reveals that they have equivalent regular secondary structure elements, as well as an almost identical spatial arrangement of the residues involved in the active site (Fig. 1). Superposition of these structures suggests that the RNase U2 residue equivalent to RNase T1 catalytically essential His 92 [5] would be His 101 [13,16] (Fig. 1). With the purpose of verifying this hypothesis, this potentially catalytic His 101 has been replaced by a Gln residue and the resulting mutant protein has been purified and characterized.

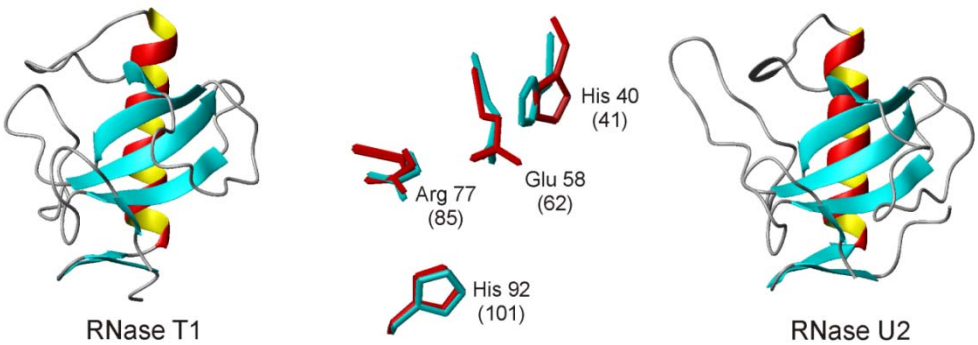

Figure 1. Representation of the structures and active site residues of RNase T1 and RNase U2. The images were generated with the MOLMOL program [42] from the atomic coordinates deposited in PDB (entries 1rnt and 1rtu, respectively). Superposition of side-chain residues corresponding to the active site of both RNase T1 (black) and RNase U2 (grey) is also represented. Numbers within brackets correspond to RNase U2.

Besides being a small enzyme, RNase U2 also shows a good number of unusual structural attributes which may be related to its particular specificity and mode of action. For example, one of its most distinctive features is the absence of any Lys residue and the presence of just a single Arg, what results in a highly acidic pI value of about 3.0 [1,17,18], and an anomalous electrophoretic behavior, even in the presence of SDS [19]. It is also a peculiar protein from a spectroscopic point of view since it contains a single but strongly quenched Trp residue [20,21]. Natural wild-type RNase U2

in solution is slowly converted upon ageing into two other isoforms containing one or two isoaspartate bonds and different secondary structure and specific enzymatic activity [17,21-23]. Finally, RNase U2 contains three disulfide bridges being only two of them conserved among most of the fungal extracellular RNases known [4,24]. Interestingly, the non-conserved one is established between Cys 1 and Cys 54, contiguous to Cys 55, also involved in another bridge with Cys 96 [1,25]. Mispairing of the disulfide network due to the presence of these two consecutive Cys residues (54 and 55) has been invoked to explain the presence of two isoforms upon recombinant production of RNase U2 in the yeast *Pichia pastoris* [4,21]. In the present work this hypothesis has been also further explored through production, purification, and characterization of a double mutant where both Cys residues 1 and 54 have been replaced by Ser.

Materials and methods

DNA Manipulations

All of the materials and reagents were of molecular biology grade. Cloning procedures and bacteria manipulations were carried out according to standard methods [26], as described previously [27,28]. *In vitro* site-directed mutagenesis using a double-stranded DNA template and two oligonucleotides annealing to the complementary strands was used to obtain the mutants as described [26]. Two different mutants were constructed: A single one where His 101 was replaced by Gln (H101Q) and a double one where Cys 1 and 54 were both substituted by Ser (C1/54S). The template employed was the plasmid pPICZαU2, used before to produce the wild-type protein in *P. pastoris* [19], or that one corresponding to the mutated Cys 1 for the double mutant. These plasmids confer resistance to the antibiotic zeocin. The mutagenic primers used are shown in Table 1. PCR amplification of the mutagenic mixture was performed in standard conditions using a mixture of Taq-Gold and Vent DNA polymerases and the Taq-extender buffer to complete the copy of the entire lengths of both template complementary DNA strands. Selection of mutants was achieved after treatment with DpnI, which specifically cleaves methylated sequences [29]. The expression constructs were obtained using the *Escherichia coli* strain DH5αF' [26-28,30]. Presence of only the mutations expected in each case was confirmed by sequencing the complete cloned cDNA at the Universidad Complutense DNA-Sequencing Facility.

Production and purification of the proteins

P. pastoris GS115 or KM71 cells carrying the corresponding plasmids were screened as described [19,21,31]. The appropriate clones were first selected on zeocin-containing agar plates and then through small-scale production experiments. Large-scale production of the recombinant proteins was carried out in buffered minimal medium as described [19,21]. The extracellular media of the corresponding cultures were exhaustively dialyzed against 20 mM piperazine, pH 6.0, and then

loaded onto DEAE-cellulose equilibrated in the same buffer. The protein was eluted in each case with a 0-0.5 M NaCl linear gradient. Fractions containing the wild-type RNase U2 or its mutant variants were pooled, dialyzed against 50 mM sodium acetate, pH 4.5, and chromatographied on a 2',5'-ADP-Sepharose (Pharmacia) column, which acts as an affinity bed for fully active RNase U2 [21]. Proteins were finally eluted with a linear gradient, 50 mM sodium acetate (pH 4.5)/50 mM Tris-HCl (pH 7.0) containing 50 mM NaCl. When purifying the H101Q mutant, volume excluded from the affinity column was also collected, concentrated, and further fractionated by means of a gel filtration step on a Bio-Gel P10 column (2 x 150 cm) equilibrated in 50 mM Tris-HCl, pH 7.0, containing 0.1 M NaCl. After both chromatographic steps, fractions containing the mutant RNases U2 were finally pooled, concentrated, dialyzed against 50 mM ammonium acetate, pH 4.5, and lyophilized.

Table 1. Mutagenic primers used to construct the RNase U2 mutants H101Q and C1/54S. The bases that change the original codon are underlined.

mutation	oligonucleotide sequence
C1S forward	5'- AAA AGA GAG GCT GAA GCT GAA TTG TCC GAC ATC CCT CAG TCC ACC AAC TGC -3'
C1S reverse	5'- GCA GTT GGT GGA CTG AGG GAT GTC GGA CAA TTC AGC TTC AGC CTC TCT TTT -3'
C54S forward	5'- GAA GCG TCT GAA GAC ATT ACT CTT TCC TGT GGA TCC GGT CCT TGG TCC GAA -3'
C54S reverse	5'- TTC GGA CCA AGG ACC GGA TCC ACA GGA AAG AGT AAT GTC TTC AGA CGC TTC -3'
H101Q forward	5'- GGA GAG TTT TGT GCA ACC GTC ACT CAA ACG GGT GCA GCT AGT TAT GAC GGC -3'
H101Q reverse	5'- GCC GTC ATA ACT AGC TGC ACC CGT TTG AGT GAC GGT TGC ACA AAA CTC TCC -3'

Electrophoretic and chemical characterization

Given the particular electrophoretic behavior of RNase U2, polyacrylamide gel electrophoresis and Western blots were performed as described before [19]. Glycoproteins and glycopeptides were detected with biotinylated-Con A lectin following a standard procedure [32,33]. Protein hydrolysis and amino acid analysis were performed according to standard procedures [27,34]. These analyses were employed to estimate extinction coefficients ($E^{0.1\%}$) and protein concentrations (Table 2). Immunodetection of the blotted proteins was performed with a rabbit polyclonal sera raised against purified recombinant wild-type RNase U2 [19].The amino-terminal sequences were determined by Edman degradation using an Applied Biosystems model 477A sequencer at the C.I.B.-C.S.I.C. (Madrid, Spain) Protein Sequencing Facility.

Table 2. - Purification yields and structural features of the different RNase U2 protein versions studied.

Protein	yield[a]	$E^{0.1\%}$ (280 nm, 1 cm)	Tm (ºC) pH 4.5	Tm (ºC) pH 7.0	NH_2-terminal sequence
WT[b]	0.95	1.80	61	50	Glu-Ala-Glu
H101Q	0.71	n.d.[c]	n.d.[c]	n.d.[c]	Glu-Ala-Glu Glu-Leu-Cys Ala-Glu-Ala
C1/54S	0.78	1.74	50	42	Glu-Leu-Ser

[a]expressed as mg of protein isolated by liter of original yeast culture.
[b][19]
[c]not determined

Spectroscopic characterization

Absorbance measurements were performed on an Uvikon 930 spectrophotometer at 100 nm/min scanning speed, at room temperature and in 1 cm optical path cells. Circular dichroism (CD) spectra were obtained on a Jasco 715 spectropolarimeter, equipped with a thermostated cell holder and a NesLab-111 circulating water bath, at 0.2 nm/s scanning speed. The instrument was calibrated with (1)-10-camphorsulfonic acid. CD spectra were recorded in cylindrical cells of 0.1 cm optical path. Mean residue weight ellipticities were expressed in units of degree x cm^2 x $dmol^{-1}$. Thermal denaturation profiles were obtained by measuring the temperature dependence of the ellipticity at 220nm in the 25–85ºC range. The temperature was continuously changed at a rate of 0.5ºC/min. Tm (temperature at the midpoint of the thermal transition) values were calculated assuming a two-state unfolding mechanism [35]. Fluorescence emission spectra were recorded on an SLM Aminco 8000 spectrofluorimeter at 25ºC using a slit width of 4 nm for both excitation and emission beams. The spectra were recorded for excitation at 275 and 295 nm and both were normalized by considering that Tyr emission above 380 nm is negligible. The Tyr contribution was calculated as the difference between the two normalized spectra. Thermostated cells with a path length of 0.2 and 1.0 cm for the excitation and emission beams, respectively, were used. The temperature was controlled by a circulating water bath. All these experiments were performed with the proteins dissolved in 50 mM sodium acetate pH 4.5.

Mass spectrometry

Protein samples were analyzed as described before [19] on an Autoflex III MALDI-TOF-TOF instrument (Bruker Daltonics, Bremen, Germany) with a smartbeam laser. The spectra were acquired using a laser power just above the ionization threshold. Samples were analysed in the positive ion detection and delayed extraction linear mode. Typically, 1000 laser shots were summed into a single

mass spectrum. External calibration was performed, using the Protein Calibration I from Bruker, covering the range from 5000 to 17000 Da. The 2,5-dihydroxy-acetophenone (2,5-DHAP) matrix solution was prepared by dissolving 7.6 mg (50 µmol) in 375 µl ethanol followed by the addition of 125 µl of 80 mM diammonium hydrogen citrate aqueous solution. For sample preparation, 2.0 µl of the sample were diluted with 2.0 µl of 2% trifluoro acetic acid aqueous solution and 2.0 µl of matrix solution. A volume of 1.0 µl of this mixture was spotted onto the stainless steel target and allowed to dry at room temperature.

***Ribonucleolytic Activity*.**

The activity of the purified proteins was assayed by using a zymogram against poly (A) in 15% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS and 0.3 mg/ml of the homopolynucleotide as previously described [19,27,28,30,36-38]. After electrophoresis, the gel was washed to eliminate the SDS, incubated at pH 4.5 for 1.5 h at 37 °C, and then stained with 0.2% (w/v) toluidine blue. The proteins exhibiting ribonuclease activity appear as colorless bands, because of degradation of the polynucleotide, after appropriate distaining. This assay is also useful to detect the presence of other RNA degrading activities in the protein samples. Volumograms of these bands (based on integrating all of the pixel intensities composing the spot) were obtained with the photo documentation system UVI-Tec (Cambridge, UK) and the software facility UVIsoft UVI band Windows Application V97.04 [19,27,28,30,38]. These data were used to quantify the activity.

Results and Discussion

Protein production and purification

In order to study the potentially catalytic role of His 101, the RNase U2 H101Q mutant was prepared, produced in *P. pastoris* and purified. Unexpectedly, no 2',5'-ADP-Sepharose binding fraction was detected but rather all the mutant protein appeared in the void volume of the column, according to its electrophoretic and immunogenic behavior (Fig. 2). This affinity chromatography step was introduced previously [4,21], when first purifying the recombinant wild-type RNase U2, in order to remove a presumably misfolded fraction of the protein with an apparent Trp fluorescence. However, now the production of H101Q in identical conditions rendered only a protein fraction unable to bind to the affinity column. The 2',5'-ADP ligand should still be recognized if the active site geometric arrangement was being preserved in the mutant. In fact, substitution of the equivalent His residue in other fungal extracellular RNases has resulted in inactive mutant proteins which still retained the native conformation [28,39]. So, this result suggested a misfolded conformation of the H101Q mutant to explain its inability to bind to the column. In order to confirm this suggestion, this excluded protein fraction was further fractionated by means of a gel filtration column, pooled and characterized (Table 2).

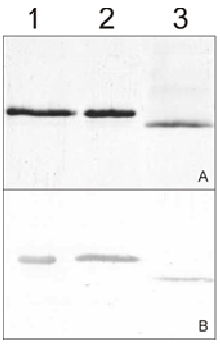

Figure 2. Electrophoretic analysis. (A) SDS-PAGE and (B) Western blot of wild type RNase U2 (1) and its C1/54S (2) and H101Q (3) mutants. All samples were boiled in the presence of 5.0 % β-mercaptoethanol before being loaded onto the gel.

Cysteine pairing isomerism involving Cys residues 1, 54, 55 and 96 has been invoked to explain the presence of a misfolded wild-type RNase U2 fraction within the extracellular media of *P. pastoris* cells harboring the pPICZαU2 plasmid [4,21]. Given the result obtained upon purification of the H101Q mutant, and now in order to study the role of the non-conserved disulfide bridge, the RNase U2 C1/54S double mutant was also produced and purified (Fig. 2). In this case, the mutant did bind to the column with no immunoreactive protein detected in the excluded fractions, suggesting that only a properly folded protein fraction was being produced, with a very similar yield than in the case of the wild-type RNase U2 (Table 2).

Structural characterization

Amino acid analyses matched those ones expected according to their cDNA-deduced amino acid sequences (data not shown). Furthermore, both protein mutants isolated were purified to electrophoretic homogeneity (Fig. 2). RNase U2 does not bind SDS under the SDS-PAGE conditions, its electrophoretic mobility being only determined by its electrostatic charge and hydrodynamic properties. Accordingly, the C1/54S mutant, lacking one disulfide bridge, exhibited a lower mobility when applied under non-reducing conditions (Fig. 3), suggesting a more relaxed conformation. In both cases studied the presence of a predominant immunoreactive band was evident confirming that a protein with the RNase U2 polypeptide primary structure was the main component in the purified preparations (Fig. 2).

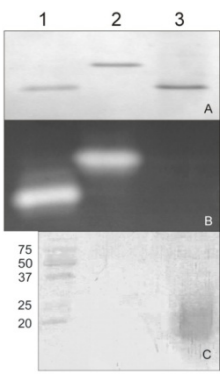

Figure 3. (A) SDS-PAGE and (B) zymogram against poly (A), at pH 4.5, of wild type RNase U2 (1) and its C1/54S (2) and H101Q (3) mutants. Prior to be applied on the gel, all samples were boiled but in the absence of β-mercaptoethanol. (C) Biotinylated-Con A staining of a Western blot where the equivalent samples had been electrotransferred. In this case, samples were reduced with 5.0% β-mercaptoethanol before being loaded onto the polyacrylamide gel. The first line on the left corresponds to pre-stained *Precision Plus Protein*™ Standard.

In wild-type RNase U2, Cys 1 impairs proper proteolytic processing of the yeast α-factor signal peptide artificially fused to the RNase U2 sequence when produced in *P. pastoris* (Fig. 4). This fact explains why the recombinant wild-type protein still retains six amino acids belonging to that signal peptide (Fig. 4 and Table 2) [16,18]. Amino-terminal sequencing revealed the presence of at least three different sequences for the H101Q sample (Table 2), corresponding to three different sites of signal-peptide proteolytic processing (Fig. 4), and confirming the inefficiency of this cleavage which is indeed heterogeneous for this mutant. On the other hand, substitution of Cys 1 by Ser rendered a protein with a single amino-terminal sequence (Table 2) corresponding to a more efficient signal-peptide cleavage. Thus, only two extra residues remain in the C1/54S mutant when compared to the natural mature protein (Fig. 3). These observations were further confirmed by MALDI-TOF analyses (Fig. 5). The results obtained matched the molecular mass expected for the C1/54S protein while they were higher and more heterogeneous for the other mutant (Fig. 5), suggesting the presence of posttranslational modifications for the H101Q RNase U2 as well as the presence of small size contaminants. Heterologous protein glycosylation in *P. pastoris* is rather frequent even if that protein is not glycosylated by its native host [40,41]. Wild-type RNase U2 and the two mutants studied do not contain an Asn-glycosylation consensus sequence. Nevertheless, it has been shown that *P. pastoris* can also add O-oligosaccharides composed of mannose to different residues of the same protein [40]. In fact, the H101Q mutant showed high degree of heterogeneous glycosylation (Fig. 3) while the wild-type and the C1/54S variant were not modified in identical culture conditions. Interestingly, the substituted His 101 is flanked by two Thr residues (Thr 100 and Thr 102) that eventually might become accessible to glycosylation upon protein misfolding. Furthermore, *P. pastoris* secretes mannans at high concentration that can non-covalently associate with heterologous proteins expressed extracellularly and copurify with them [41], which may explain the presence of many of the small size contaminants observed in the H101Q MS spectrum. In good agreement with all results shown above, far-UV CD spectra of wild-type RNase U2 [19] and its C1/54S variant were indistinguishable (Fig. 6), while the H101Q mutant displayed a much different spectrum, consistent with high content of random secondary structure (Fig. 6).

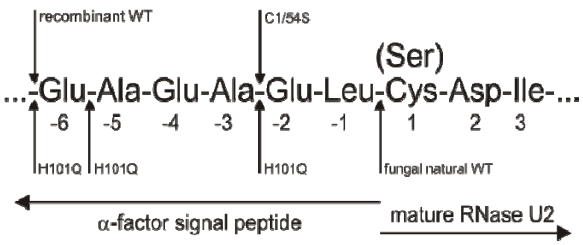

Figure 4. Scheme showing the different signal peptide cleavage processing sites found for the different recombinant proteins studied according to their amino-terminal amino acid sequences.

One of the most distinct spectral features of RNase U2 is the low fluorescence emission above 340 nm besides the existence of a Trp residue. Consequently, the C1/54S mutant protein showed the characteristic fungal natural RNase U2 fluorescence emission spectra centered around 300 nm when excited at 275 nm (Fig. 7). On the other hand, quantum yields were much higher and Trp fluorescence emission was much more evident in the H101Q preparation (Fig. 7), as it had been

observed before with the excluded protein fraction of the wild type RNase U2 [4,21], and in good agreement with a misfolded conformation.

Thermal denaturation profiles of both wild-type and C1/54S RNases U2 (Fig. 5) confirmed that the mutant protein displayed a native conformation. The absence of the disulfide bridge was however evidenced by its much lower Tm values when compared to the wild-type protein (Table 2).

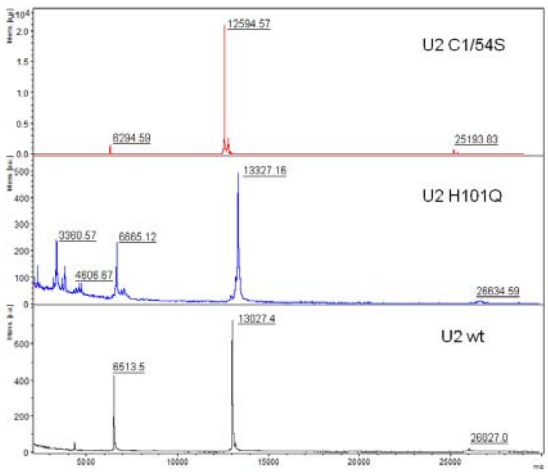

Figure 5. Maldi-TOF analyses of wild-type RNase U2 and its mutants C1/54S and H101Q.

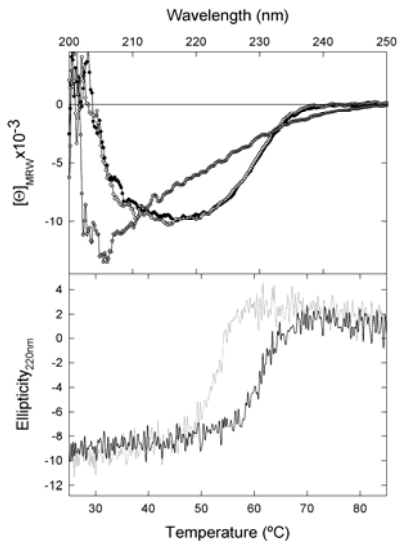

Figure 6. Spectroscopic characterization. (A) Far-UV circular dichroism spectra of wild-type (●), C1/54S (○), and H101Q (⊙) recombinant versions of RNase U2 (B) Thermal denaturation profiles at pH 4.5 for wild-type RNase U2 (black) and its mutant C1/54S (grey) measured by recording the ellipticity change at 220nm (Θ_{220}) vs temperature. Mean residue weight ellipticity ($[\Theta]_{MRW}$ or Θ_{220}) is expressed in deg cm^2 $dmol^{-1}$.

In summary, the structural characterization revealed that the C1/54S mutant protein, capable of binding to the 2',5'-ADP-Sepharose, exhibited the wild-type native conformation but with a much reduced thermostability and a shorter amino-terminal remain of the artificially fused signal peptide. On the other hand, the H101Q variant was not retained by the affinity column and displayed structural and spectroscopic features that were not compatible with the natural fungal RNase U2 native conformation.

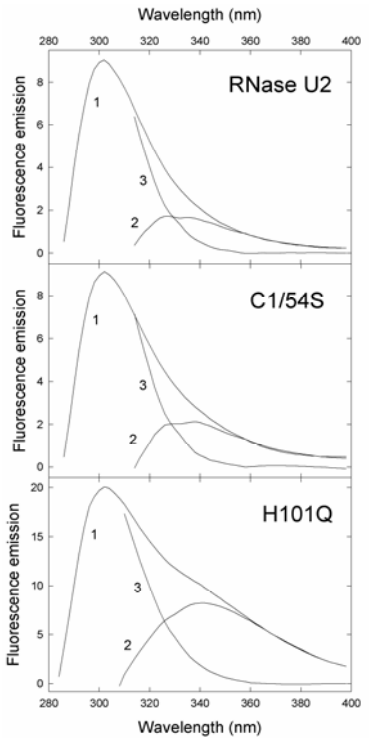

Figure 7. Fluorescence emission spectra of wild-type RNase U2 and the mutants C1/54S and H101Q. All spectra were recorded using proteins with identical 280 nm-absorbance values. Spectra labeled '1' resulted from excitation at 275 nm and spectra labeled '2' from excitation at 295 nm (tryptophan contribution). These spectra were normalized at wavelengths above 380 nm. Spectra '3' (tyrosine contribution) were calculated by subtracting spectra '2' from spectra '1'. Fluorescence emission units were arbitrary.

Enzymatic characterization

Zymogram assays showed that the C1/54S mutant did cleave the homopolynucleotide poly (A) to a comparable extent (60%) as the native recombinant wild-type RNase U2 at pH 4.5, a unique feature of this enzyme (Fig. 3). In agreement with the structural data discussed above, the H101Q mutant was devoid of detectable enzymatic activity when assayed under identical conditions (Fig. 3).

Conclusions

Structural comparisons suggest RNase U2 His 101 as the best candidate residue to fulfill the role of general acid during the transphosphorylation step leading to RNA cleavage. Unexpectedly, mutation of this residue by Gln strongly supports the major conclusion that this residue is essential for proper protein folding upon production in *P. pastoris*. On the other hand, the non-conserved disulfide bond very clearly influences the stability of the protein, since its elimination results in a native and fully active but less stable mutant RNase U2, as evidenced by the thermostability experiments. However, the presence of Cys 1 leads to a deficient removal of the yeast α–factor signal peptide used to induce its secretion to the extracellular medium. Both Cys 1 and 54 do also seem to allow a percentage of incorrect disulfide bridges arrangement resulting in the production of a significant amount of wild-type protein molecules exhibiting a non-native folding pattern and therefore unable to bind to the affinity column employed for their purification.

Acknowledgements

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Resultados B

IMPLICACIÓN BIOLÓGICA DE LA ACCIÓN DE LAS RIBOTOXINAS EN LA FUNCIONALIDAD DEL RIBOSOMA

B1. La ruptura del lazo sarcina/ricina en el rRNA 23S inhibe la translocación dependiente de EF-G.

Las ribotoxinas son potentes inhibidores de la biosíntesis de proteínas y son capaces de inactivar los ribosomas de diversos organismos al hidrolizar un único enlace fosfodiéster en una región del rRNA 23S conocida como lazo sarcina/ricina (SRL). Esta región es esencial en la funcionalidad del ribosoma y por eso se encuentra universalmente conservada. El SRL forma parte del sitio de interacción de los factores de elongación, EF-G y EF-Tu en el caso de las bacterias, con el rRNA de la subunidad mayor del ribosoma. En cada ciclo de elongación, en el que la cadena polipeptídica aumenta en un aminoácido, ocurren tres etapas. En la primera, EF-Tu•GTP forma un complejo ternario con un aminoacil-tRNA, que se une al ribosoma. Al reconocerse un codón adecuado el factor de elongación hidroliza el GTP colocándose el aminoacil-tRNA en el sitio A. En la segunda, se produce la reacción peptidil transferasa, quedando un tRNA desacilado en el sitio P y la cadena peptídica creciente, unida a otro tRNA, en el sitio A. La última etapa de cada ciclo es la translocación, catalizada por el EF-G, en la que el tRNA desacilado y el peptidil-tRNA, junto con el mRNA, se desplazan para comenzar un nuevo ciclo, hasta los sitios E y P, respectivamente. En este trabajo se ha observado que la ruptura del SRL por parte de la α-sarcina provoca un efecto moderado en la unión del complejo ternario al ribosoma pero reduce la unión del EF-G e inhibe significativamente su actividad para hidrolizar GTP. Ello impide la translocación, con lo que la inhibición de la biosíntesis de proteínas queda completamente explicada. No obstante, los resultados también sugieren que la integridad del SRL no es esencial para el movimiento del mRNA y los tRNAs *per se*, pero sí para la unión del EF-G que contribuye a facilitar estos desplazamientos.

Trabajo B1: García-Ortega L, **Álvarez-García E**, Gavilanes JG, Martínez del Pozo Á y Joseph S (2009) Cleavage of the sarcin-ricin loop of 23S rRNA differentially affects EF-G and EF-Tu binding. Enviado a RNA.

Cleavage of the Sarcin-Ricin Loop of 23S rRNA Differentially Affects EF-G and EF-Tu Binding

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Abstract

Ribotoxins are potent inhibitors of protein biosynthesis and inactivate ribosomes from a variety of organisms. The ribotoxin α-sarcin cleaves the large 23S ribosomal RNA (rRNA) at the universally conserved sarcin-ricin loop (SRL) leading to complete inactivation of the ribosome and cellular death. The SRL is important for binding translation factors that hydrolyze GTP, but its precise role is not yet understood. We studied the effect of α-sarcin on defined steps of translation by the bacterial ribosome. α-Sarcin-treated ribosomes showed no defects in mRNA and tRNA binding, peptide-bond formation and sparsomycin-dependent translocation. Cleavage of SRL slightly affected binding of elongation factor Tu ternary complex (EF-Tu●GTP●tRNA) to the ribosome. In contrast, the activity of elongation factor G (EF-G) was strongly impaired in α-sarcin-treated ribosomes. Importantly, cleavage of SRL inhibited EF-G binding, and consequently GTP hydrolysis and mRNA-tRNA translocation. These results suggest that the SRL is more critical for binding EF-G than EF-Tu ternary complex to the ribosome. The SRL may interact differentially with EF-Tu and EF-G to dynamically modulate their activity during the elongation cycle of protein synthesis.

Introduction

Ribotoxins are a family of toxic extracellular fungal ribonucleases (RNases) that exert an exquisite ribonucleolytic activity on the larger rRNA, leading to protein synthesis inhibition and cell death by apoptosis (Lacadena et al. 2007). α-Sarcin, a well studied ribotoxin, cleaves the phosphodiester bond between G2661-A2662 located in helix 95 of 23S rRNA (Schindler and Davies 1977; Endo and Wool 1982; Hausner et al. 1987; Lacadena et al. 2007) (Figure 1A and 1B). Helix 95 is also targeted by ricin, a N-glycosidase that inactivates the ribosome by depurinating A2660 (Endo et al. 1987). Hence helix 95 of 23S rRNA is known as the sarcin-ricin loop (SRL). The SRL comprises one of the longest stretches of universally conserved rRNA sequence indicating a crucial role in protein synthesis (2654-2665 in *E. coli* 23S rRNA) (Egebjerg et al. 1990; Gutell et al 1992). Consistent with an important functional role, mutations or deletions in the SRL sequence result in lethal effects on cell growth (Macbeth and Wool 1999; Chan et al. 2000, Lancaster et al. 2008).

The SRL together with the GTPase-associated center (GAC, helices 43 and 44 of 23S rRNA) form the main interaction site for EF-G and EF-Tu with the rRNA in the large ribosomal subunit (Moazed et al. 1988; Valle et al. 2003a, 2003b). More recently, studies have shown that *E. coli* initiation factor 2 (IF2) and release factor 3 (RF3) interact with the same region of the ribosome (La Teana et al. 2001; Klaholz et al. 2004). In general, all these proteins are GTPases that bind to the ribosome, hydrolyze GTP and undergo conformational changes before dissociating from the ribosome. A challenging problem is to understand how the ribosome regulates the association of specific factors during defined steps of protein biosynthesis.

Three major steps occur during protein elongation (Wintermeyer et al. 2004). First, a ternary complex formed by EF-Tu, GTP and the cognate aminoacyl-tRNA binds to the A site of the ribosome carrying either an initiator fMet-tRNA^{fMet} or peptidyl-tRNA in P site. EF-Tu●GDP dissociates from the ribosome and tRNA is accommodated into the A site. In the second step, the ribosome catalyzes the peptidyl transferase reaction, resulting in a deacylated tRNA at the P site and a peptidyl-tRNA at the A site. The cycle is completed by the translocation of the tRNAs-mRNA complex catalyzed by EF-G. After translocation and release of EF-G, the ribosome repeats the elongation cycle until a stop codon is encountered. The entry of a stop codon into the A site signals the termination of protein synthesis.

Recent biochemical studies as well as structural information from cryo-EM images have helped to elucidate the role of EF-Tu and EF-G. During tRNA selection by the ribosome, codon recognition triggers GTP hydrolysis by EF-Tu that catalyzes the accommodation of tRNA into the A site (Pape et al. 1999). Interestingly, GTP hydrolysis by EF-G is not directly coupled to the movement of the tRNAs and mRNA (Rodnina et al. 1997). In this case, GTP-hydrolysis induces conformational changes in the ribosome, called “unlocking”, that allow the disruption of the interactions between the A site tRNA and the 30S subunit decoding center promoting a rapid translocation of the mRNA-tRNA complex (Frank and Agrawal 2000; Valle et al. 2003b; Taylor et al. 2007). Moreover, it has been suggested that GTP hydrolysis by EF-Tu and EF-G are triggered by ribosomes in two different

conformational states corresponding to the different steps in the elongation cycle (Valle et al. 2003a, 2003b; Sergiev et al. 2005). How binding of EF-G and EF-Tu to the ribosome triggers GTP hydrolysis is still not clear. Since the SRL interacts intimately with the GTPase domain of EF-Tu and EF-G, it is likely to play a crucial role during GTP hydrolysis -(Moazed et al. 1988; Connell et al. 2007).

Interestingly, Nierhaus and co-workers showed that cleavage of the SRL by ?-sarcin modestly affected the binding of EF-Tu ternary complex, while the binding of EF-G and translocation were strongly inhibited (Hausner et al. 1987). In this study, however, just more than 50% of the ribosomes were estimated to be cleaved by α-sarcin and it was not possible to directly correlate the ribosomal activity with the extent of cleavage. Furthermore, recent studies suggest that the SRL may participate in additional steps catalyzed by EF-G and EF-Tu. For example, G2655C mutation in the SRL which was lethal and strongly inhibited translocation, only moderately affected binding of EF-G and GTPase activity (Leonov et al. 2003). Additionally, a single-molecule study showed that ribosomes treated with the α-sarcin homolog, restrictocin, were able to bind ternary complex and progress up to the GTPase-activated state (Blanchard et al. 2004). Thus, in some instances, EF-G and EF-Tu can bind to the ribosome with an inactive SRL.

In order to further explore the importance of the SRL for the elongation step of protein synthesis, we cleaved the SRL with α-sarcin. Conditions were optimized to get a more uniform extent of cleavage by α-sarcin and the level of cleavage was quantitated precisely by primer extension analysis. Consistent with the earlier study (Hausner et al. 1987), our results show that cleavage of the SRL has modest effects on ternary complex binding, while more significantly affecting EF-G binding, GTP hydrolysis and translocation. These results suggest that, compared to EF-Tu ternary complex, EF-G requires an intact SRL for stably binding to the ribosome. Interestingly, EF-G-independent translocation, induced by sparsomycin, is not affected indicating that the SRL is not required for the movement of the mRNA-tRNA complex in the ribosome.

Results

Inactivation of E. coli ribosomes by α-sarcin

It is well known that *E. coli* ribosomes are less susceptible to α-sarcin cleavage than their eukaryotic counterparts (Schindler and Davies 1977; Endo and Wool 1982; Hausner et al. 1987). Previous studies have reported inactivation of *E. coli* ribosome preparations ranging from 10 to 50%. Thus, our first goal was to optimize the inactivation procedure and minimize the contamination with intact ribosomes. Consequently, different buffers, concentration of reagents and length of time for the cleavage reaction were assayed. Primer extension was used to quantify the extent of cleavage and agarose gel electrophoresis was used to analyze the specificity of the cleavage and the integrity of the rRNA after treatment with the toxin (Figure 1C and 1D). The buffers tested were variations of the standard polymix buffer (buffer A in Figure 1C) since this would be the one employed in

subsequent functional assays (Bartetzko and Nierhaus 1988). Previous reports indicated that the presence of millimolar concentrations of mono or divalent cations inhibited the ribonucleolytic activity of α-sarcin (Endo et al. 1983; Martínez del Pozo et al. 1989; Korennykh et al. 2006). Therefore, we tested polymix buffer containing different concentration of magnesium and polyamines. We tested buffer A without polyamines (buffer B), and decreased the magnesium concentration to 2 mM (buffer C) or to 0 mM (buffer D) by adding EDTA (see Materials and Methods). We obtained 75% specifically cleaved ribosomes in buffer C, which contained 2 mM magnesium, no polyamines and 0.3 μM α-sarcin. Increasing the α-sarcin concentration or completely eliminating magnesium (buffer D) in the reaction resulted in additional cleavages other than that producing the specific -fragment (Figure 1D). Thus, in our hands the best conditions gave 75% of specifically cleaved ribosomes and these were used for future experiments (Figure 1C). Ribosome requires a minimum concentration of magnesium and polyamines (Blaha et al. 2000), so these were restored after -sarcin treatment for functional assays (Bartetzko and Nierhaus 1988).

Interestingly, the most efficient conditions gave a slightly different specificity with respect to the position of cleavage in the 23S rRNA. The expected cleavage was at the 3' side of G2661 resulting in the primer extension a stop at position A2662 (Figure 1C). However, in addition, a band appeared at position G2661 due to cleavage at the 3' side of A2660. Close inspection of previous published results revealed a similar heterogeneity in the cleavage of *E. coli* ribosomes (Macbeth and Wool 1999). Most probably this second cleavage site corresponded to a less specific action of the toxin since the conditions employed were very drastic, which might have affected the conformation of the SRL.

In vitro translation is inhibited by cleaving the SRL

Protein composition of the α-sarcin treated ribosomes was analyzed to determine whether cleavage of the SRL caused loss of ribosomal proteins. This was accomplished by filtering α-sarcin treated ribosomes to separate possible unbound ribosomal proteins. The filtered fractions were then analyzed by SDS-PAGE (Figure 2A). Both untreated control ribosomes and α-sarcin treated ribosomes showed similar levels of ribosomal proteins indicating no significant loss of proteins due to cleavage of the SRL. Although the protein electrophoresis cannot resolve all the ribosomal proteins, proteins L6, L11 and L14 that bind close to the SRL and are more likely to be affected by the action of α-sarcin, have molecular weights within the resolution range of the gel. These proteins were present at similar levels in the α-sarcin treated ribosomes. Supporting this conclusion, no proteins were found in the filtrate besides α-sarcin.

Next, the ability of α-sarcin treated ribosomes to bind aminoacylated tRNAs to the A site without the help of EF-Tu was determined. Ribosomes programmed with a defined mRNA and f-[^{35}S]Met-tRNA^{fMet} in the P site were incubated with Phe-tRNA^{Phe}. The amount of f-[^{35}S]Met-Phe dipeptide formed corresponds to the amount of Phe-tRNA^{Phe} bound to the A site. Dipeptides formed by the ribosome were separated by electrophoretic TLC (eTLC) and quantitated (Figure 2B). The extent of dipeptide formed was similar with untreated control ribosomes and α-sarcin treated

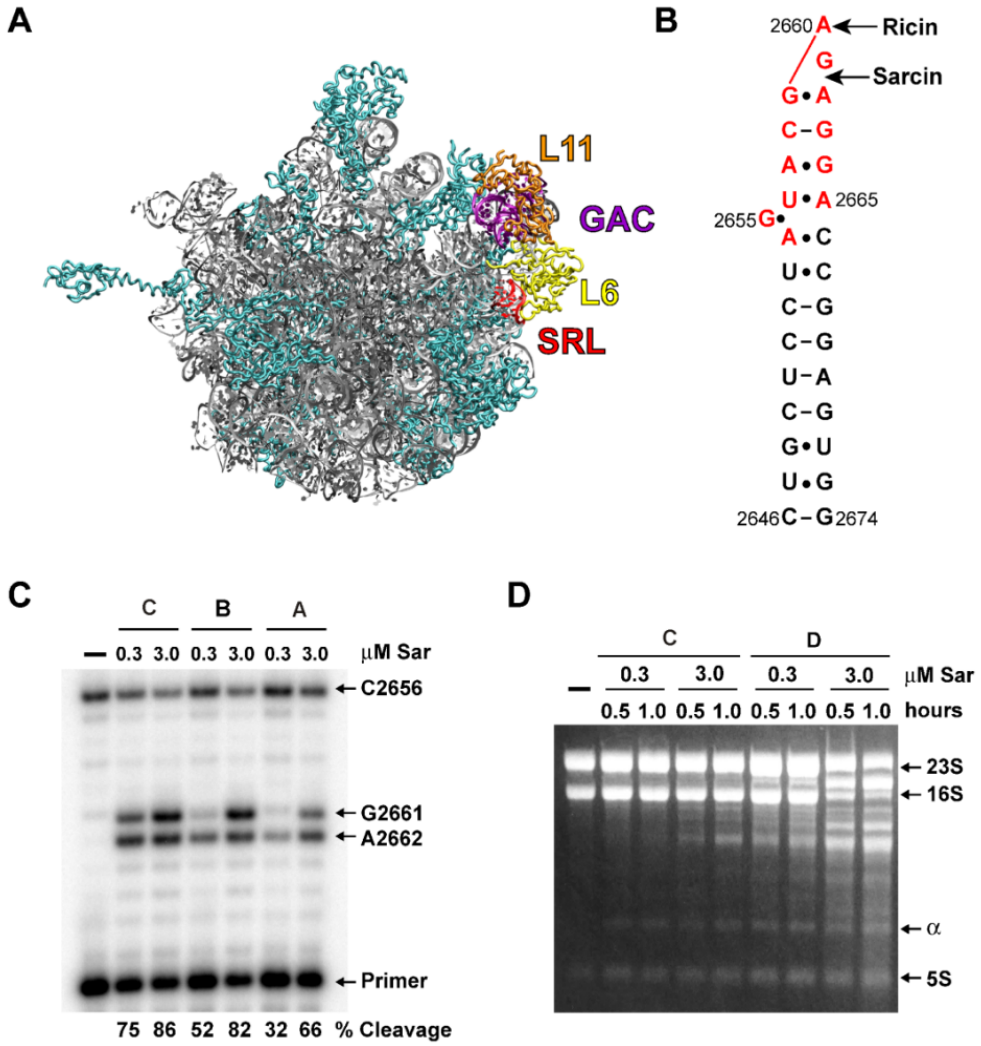

Figure 1. Cleavage of *E. coli* ribosomes by α-sarcin. (A) The x-ray crystal structure of the *E. coli* 50S ribosomal subunit (PDB accession code 2aw4). The rRNAs are represented by the grey ribbons and the ribosomal proteins by cyan tubes. Indicated are the SRL (red), GAC (purple), ribosomal proteins L6 (yellow) and L11 (orange). (B) Secondary structure of the SRL. The universally conserved bases in the SRL are shown in red. The sites of cleavage by α-sarcin and modification by ricin are indicated by the arrows. (C) Primer extension analysis of the SRL cleavage after rRNA extraction. Cleavage of *E. coli* ribosomes in buffers A (polymix buffer), B (A minus polyamines) and C (B + 6mM EDTA) for 30 min with 0.3 or 3 µM α-sarcin. Control reaction without α-sarcin is indicated by (-). The arrows indicate the position in the 23S rRNA where the reverse transcription stops. The cleavage efficiency is indicated below the lanes. (D) Agarose gel electrophoresis of rRNAs extracted from α-sarcin-treated ribosomes. The cleavage reaction was performed in buffers C and D (B + 8 mM EDTA) with 0.3 or 3 µM α-sarcin and the reaction was incubated for 0.5 or 1 hour as indicated above the lanes. Control reaction without α-sarcin is indicated by (-). 23S, 16S, 5S rRNA molecules and α-fragment are indicated by the arrows.

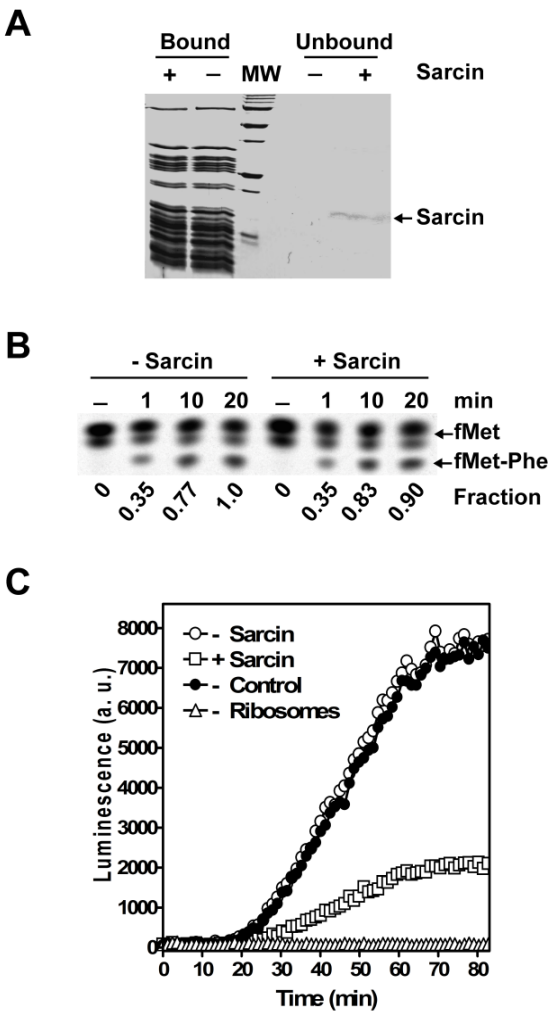

Figure 2. Effect of α-sarcin cleavage on ribosome structure and activity. (A) Protein profile of untreated control (-) and α-sarcin-treated (+) ribosomes. Proteins bound to the ribosome (bound) and free in solution (unbound) were separated by filtration and analyzed by SDS-PAGE. Position of α-sarcin (17,000 Da) is indicated by the arrow. MW represents protein standards from 250 to 10 KDa. (B) Non-enzymatic binding of tRNA and peptidyl transferase reaction. Time course of dipeptide formation with untreated (-Sarcin) and α-sarcin-treated ribosomes (+ Sarcin). The products were separated by electrophoretic TLC and the dipeptide formed (fMet-Phe) is indicated by the arrow. Normalized fraction of dipeptide obtained is indicated below the lanes. (C) *In vitro* translation. Synthesis of luciferase reporter enzyme by untreated ribosomes (open circles) and ribosomes cleaved with α-sarcin (open squares). A control reaction with α-sarcin added to the translation mixture was performed for any non-specific activity (closed circles). In addition, a reaction without ribosomes was used as a negative control (open triangles).

ribosomes. Thus, the intrinsic ability of the ribosomes to bind mRNA and tRNAs and catalyze peptide bond formation in a factor-independent manner was not impaired by cleaving the SRL.

The activity of α-sarcin treated ribosomes was further studied using an *in vitro* translation assay, which monitors the synthesis of the reporter protein *Renilla* luciferase in a cell-free system (Figure 2C). When ribosomes were treated with α-sarcin before being added to the translation mixture, 75% inhibition of luciferase biosynthesis was observed in agreement with the efficiency of the cleavage. The addition of α-sarcin to the translation mixture without previous inactivation of the ribosomes did not affect the reaction indicating that the final concentration of α-sarcin employed showed no degradation of rRNAs, mRNAs and tRNAs.

EF-Tu binding is not completely inhibited by cleaving the SRL

A previous study showed that cleaving the SRL did not affect the initial binding of the ternary complex to the ribosome but stalled the accommodation of the tRNA into the A site (Blanchard et al. 2004). We tested binding of the ternary complex to the ribosome using the dipeptide assay described above (Figure 3A). In order to ensure EF-Tu-dependent tRNA binding, a 3-fold molar excess of EF-Tu over Phe-tRNA^{Phe} was used and the reaction was allowed to proceed for a short time (30 seconds). This permited discrimination of the EF-Tu-dependent tRNA binding from the much slower EF-Tu-independent tRNA binding. α-Sarcin-treated ribosomes showed about 35% activity compared to the untreated control ribosomes (after subtracting 25% background from the intact ribosomes in the α-sarcin reaction). This indicated that cleavage of the SRL partially allowed ternary complex functionality although it was possible that the rates of tRNA accommodation and peptide bond formation were significantly affected when the SRL was cleaved, parameters not tested here.

To further analyze the binding of ternary complex, we used a centrifugal filtration method to separate free EF-Tu from EF-Tu bound to the ribosome (Wilson and Nechifor 2004). In order to stabilize the binding, a non-hydrolyzable GTP analog (GDPNP) or an inactive EF-Tu mutant (EF-Tu H84A) (Daviter et al. 2003) were used in the binding assay. EF-Tu bound to the ribosome was analyzed by SDS-PAGE (Figure 3B). α-Sarcin-cleaved ribosomes could bind the GTPase inactive ternary complexes although at lower levels compared to the untreated ribosomes. Similarly, binding of EF-Tu●GDPNP was slightly reduced with α-sarcin-treated ribosomes. Overall, our results showed that cleavage of the SRL moderately reduced ternary complex binding and progress of the aminoacyl tRNA to the peptidyl transferase reaction.

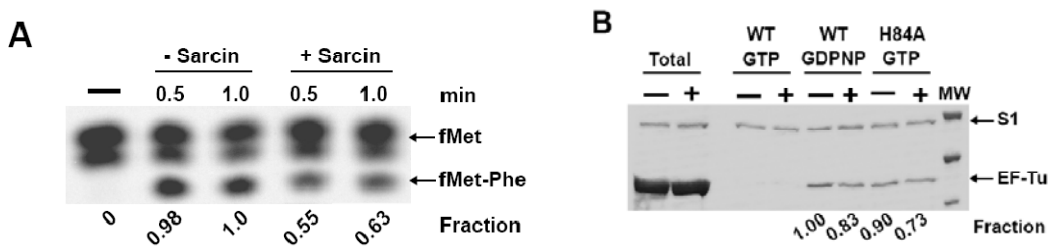

Figure 3. Interaction of EF-Tu Ternary complex with the ribosome. (A) Binding of EF-Tu●GTP●Phe-tRNA^{Phe} to the A site and peptide bond formation analyzed by electrophoretic TLC. Untreated control ribosomes (- Sarcin) and α-sarcin-treated ribosomes (+ Sarcin) were incubated with EF-Tu ternary complex for the indicated time. The dipeptide formed (fMet-Phe) is indicated by the arrow. Normalized value of dipeptide formed is indicated below the lanes. (B) Binding of EF-Tu ternary complex to ribosomes analyzed by filtration and SDS-PAGE. WT or H84A mutant EF-Tu was bound to the ribosome in the presence of GTP or GDPNP and kirromycin. Control untreated ribosomes and α-sarcin-treated ribosomes are indicated by (-) and (+), respectively. Lanes: Total, samples before filtration; MW, protein molecular weight standards of 75, 50 and 37 KDa. The position of EF-Tu and the ribosomal protein S1 are indicated by arrows. Normalized value of EF-Tu bound to the ribosome is indicated below the lanes.

Translocation catalyzed by EF-G is drastically inhibited by cleaving the SRL

The effect of cleaving the SRL with α-sarcin on EF-G-dependent translocation was next analyzed. Pre-translocation complexes were formed with either deacylated or aminoacylated tRNAs in the P and A sites. Toeprinting assay showed that α-sarcin treated ribosomes formed pre-translocation complexes with similar efficiency as control ribosomes (Figure 4A). Translocation was triggered by adding a large excess of EF-G●GTP to the pre-translocation complex and movement of mRNA-tRNA complex was monitored by the toeprinting assay (Joseph and Noller 1998). Untreated ribosomes translocated efficiently, in contrast, α-sarcin-treated ribosomes translocated poorly (Figure 4A). The background translocation observed could be attributed to the small amount of intact ribosomes present after α-sarcin treatment. Consistent with this idea, increasing the amount of tRNAs, EF-G●GTP or time of the reaction did not improve the extent of translocation (data not shown).

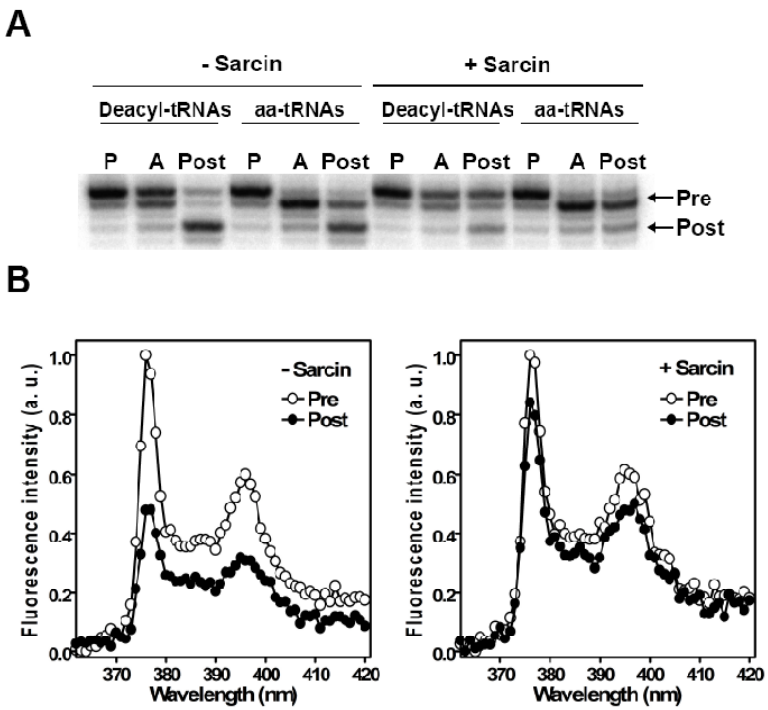

Figure 4. EF-G-dependent translocation. (A) Toeprinting analysis of translocation. Untreated control ribosomes (- Sarcin) and α-sarcin-treated ribosomes (+ Sarcin) were incubated with mRNA and either deacylated tRNAs (deacyl-tRNAs) or aminoacylated tRNAs (aa-tRNAs) to form pre-translocation complexes. Lanes: P, ribosome with $tRNA^{fMet}$ in P site; A, pre-translocation complex with $tRNA^{fMet}$ in the P and $tRNA^{Phe}$ in the A site; Post, post-translocation complex formed by the addition of EF-G-GTP to the pre-translocation complex. The toeprints corresponding to the pre-translocation complex (Pre) and the post-translocation complex (Post) are indicated by the arrows. (B) Translocation monitored by a fluorescence-based assay. Pre-translocation complex contained fMet-$tRNA^{fMet}$ in the P site and Phe-$tRNA^{Phe}$ in the A site. Post-translocation complex was formed by incubating the pre-translocation complex with EF-G and GTP. Emission spectra of pre- (open circles) and post-translocation complexes (closed circles). The decrease in pyrene emission at 376 nm was used to quantify the extent of EF-G-dependent translocation. Left panel, untreated control ribosomes; right panel, α-sarcin-treated ribosomes.

We corroborated this result with another, fluorescence-based, assay to monitor EF-G-dependent translocation (Studer et al. 2003). In this assay, translocation of the mRNA-tRNA complex causes a decrease in fluorescence intensity of the pyrene probe attached to the 3' end of a synthetic mRNA. Again, α-sarcin-treated ribosomes showed only a 25% decrease in fluorescence intensity compared to the untreated control ribosomes in the presence of a large excess of EF-G (Figure 4B). The background fluorescence intensity correlated with the amount of intact ribosomes present after treatment with α-sarcin. Thus, both the toeprinting data and the fluorescence-based assay for translocation showed that cleavage of the SRL strongly inhibited EF-G-dependent translocation.

To identify the step(s) in translocation that was inhibited by cleavage of the SRL, we tested EF-G-independent translocation. The antibiotic sparsomycin can induce translocation of the mRNA-tRNA complex, albeit at a slower rate than EF-G (Fredrick and Noller 2003). Sparsomycin-dependent translocation was monitored by toeprinting (Figure 5). Ribosomes treated with α-sarcin showed the same rate of translocation as the untreated control ribosomes. In contrast, experiments done in parallel to monitor the time course of EF-G-dependent translocation showed significant inhibition with the α-sarcin-treated ribosomes (Figure 5). These results suggested that the movement of the mRNA-tRNA within the ribosome was not affected by cleaving the SRL, but some step(s) specific to the EF-G catalyzed process was inhibited.

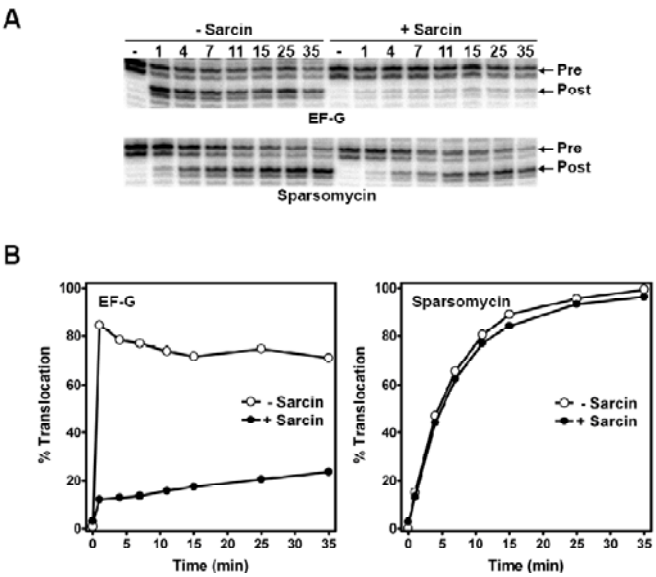

Figure 5. Sparsomycin-dependent translocation. (A) Time courses of translocation induced by EF-G (upper panel) or sparsomycin (lower panel), analyzed by toeprinting. Pre- and post-translocation toeprint bands are indicated by arrows. – lane, no EF-G or sparsomycin added; – Sarcin, untreated ribosomes; + Sarcin, α-sarcin-treated ribosomes. (B) Graphical representation of data in (A). Data were normalized to the maximum translocation value for untreated ribosomes. Open circles, untreated ribosomes; closed circles, ribosomes treated with α-sarcin. Left panel, EF-G-dependent translocation; right panel, sparsomycin-dependent translocation.

EF-G binding and GTPase activity are impaired by cleaving the SRL

Binding of EF-G to the ribosome was next tested using the filtration method described above for EF-Tu. Stable EF-G binding was observed with GDPNP and the untreated ribosomes (Figure 6A). In contrast, reduced EF-G binding was observed with the α-sarcin-treated ribosomes which was consistent with the background of intact ribosomes. Increasing the ratio of EF-G over ribosomes did not improve binding (data not shown). Thus, cleavage of the SRL reduced the affinity of EF-G for the ribosome.

Finally, we tested the ability of EF-G to hydrolyze GTP in the presence of vacant ribosomes (Figure 6B). Time course experiments were performed under multiple turnover conditions with untreated and α-sarcin-treated ribosomes mixed with varying concentrations of EF-G. The kinetic constants were extracted from the study of the dependence of initial velocities of GTP hydrolysis versus EF-G concentration of three independent experiments (Saarma et al. 1997; Savelsbergh et al. 2005; Seo et al. 2006; Nechifor et al. 2007). Parameters for the SRL cleaved ribosomes were calculated by taking into account the presence of 25% intact ribosomes after α-sarcin treatment (see Materials and Methods) (Saarma et al. 1997). We observed almost a 50-fold defect in k_{cat}/K_M for GTP hydrolysis by EF-G with the SRL cleaved ribosomes (Table I). Although the experimental data showed substantial errors, especially in K_M, a dramatic defect in GTP hydrolysis was observed when ribosomes were treated with sarcin (Figure 6B). This defect in GTPase activity was consistent with the impaired binding of EF-G to the SRL cleaved ribosomes.

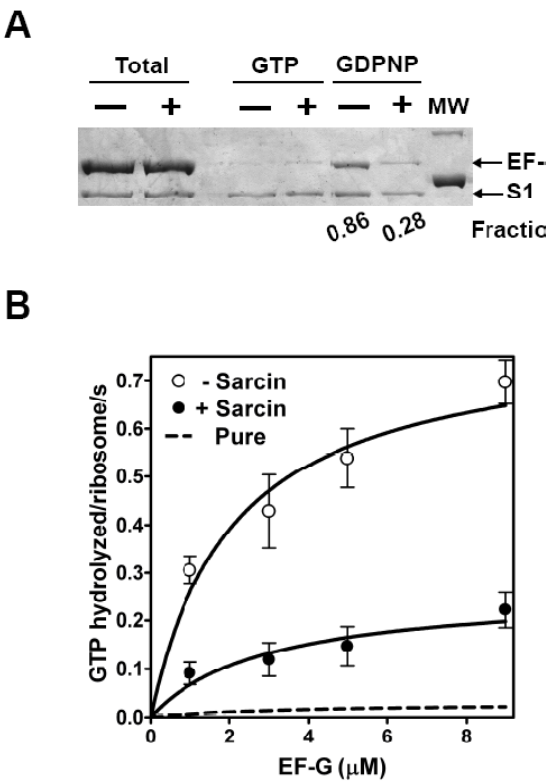

Figure 6. Interaction of EF-G with the ribosome. (A) Binding of EF-G to vacant ribosomes analyzed by filtration and SDS-PAGE. EF-G in the presence of GTP or GDPNP was used. (-), untreated ribosomes; (+), α-sarcin-treated ribosomes. Total indicates samples before separating unbound proteins. MW indicates protein standards of 100 and 75 KDa. The position of EF-G and ribosomal protein S1 are indicated by arrows. EF-G fraction bound to ribosomes is indicated below the lanes. (B) Rate of GTP hydrolysis versus EF-G concentration. Open circles, untreated ribosomes; closed circles, α-sarcin-treated ribosomes. Three independent experiments were averaged and the data fitted to a Michaelis-Menten equation. Dotted curve is theoretically obtained from the calculated kinetic constants in Table I for a population containing only cleaved ribosomes (Pure).

Table I. GTP hydrolysis by EF-G in vacant ribosomes

Ribosome	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} s^{-1})
- Sarcin	0.80 ± 0.12	2.08 ± 0.94	0.38
+ Sarcin	0.26 ± 0.09	2.90 ± 2.55	0.09
Pure	0.03	3.41	0.008

- Sarcin, ribosomes not treated with α-sarcin. + Sarcin, ribosomes treated with α-sarcin, having 25% of intact ribosomes. Pure, kinetic constants calculated for a pure population of cleaved ribosomes (Saarma et al. 1997).

Discussion

Ribotoxins are an interesting family of enzymes due to their powerful and deadly activity against numerous types of living cells. Besides their ability of reaching the cytoplasm, the ribosome inactivation that results from their ribonucleolytic activity has stimulated a lot of research in the field.

Prokaryotic SRL cleavage by α-sarcin

Ribotoxins do not affect in the same manner ribosomes from different origins even with the conserved SRL as the substrate (Schindler and Davies 1977; Miller and Bodley 1991). It has been proposed that the presence of C2666 instead of the corresponding eukaryotic G at the equivalent SRL position might explain the lower susceptibility of *E. coli* ribosomes against α-sarcin cleavage. Previous studies with small synthetic oligoribonucleotides mimicking the SRL sequence have suggested that there are at least two SRL areas that are recognized by this toxin, the GAGA tetraloop and the bulged G2655 (Figure 1B), indicating that the primary determinant for recognition is, more than the sequence, the SRL conformation (Moazed et al. 1988; Wool et al. 1992; Glück and Wool 1996; Correll et al. 1999; Pérez-Cañadillas et al. 2000; Correll et al. 2003; Correll and Swinger 2003). Furthermore, crystal structures with different analogs that mimicked the SRL revealed that the residue equivalent to A2662 docked in the active site of the ribotoxin instead of the expected G2661, leading the authors to propose an alternative pathway for cleavage (Yang et al. 2001).

Our observation that two contiguous phosphodiester bonds within the prokaryotic SRL are susceptible to the action of the toxin (Figure 1C), not only agrees with previous published data, some of them unnoticed, but also reveals that the recognition of intact ribosomes by ribotoxins might be more complex. In fact, a recent hypothesis focuses on the ability of ribotoxins to interact with other

elements of the ribosomal machinery, such as the proteins L6 and L14, in order to access the SRL (García-Ortega et al. 2002; García-Mayoral et al. 2005). In addition, electrostatics has an essential role in locating the ribotoxin on the ribosome surface contributing to its high affinity (Korennykh et al. 2006). In this study, relaxed specificity of ribotoxins is observed when trying to improve their activity against prokaryotic ribosomes by using certain conditions of magnesium and polyamines concentration. These counter ions directly affect the stability and conformation of the ribosomes, which could be the cause for the second cleavage site in the SRL by α-sarcin.

Effect of SRL cleavage on EF-G-dependent translocation

The SRL is not essential for factor-independent peptide synthesis (Chan and Wool 2008). In contrast, elongation factor related functions have been observed to be defective after ribotoxin treatment of different ribosomes. In general, the binding of either EF-Tu ternary complex or EF-G (or their eukaryotic homologs) had been concluded as the main defect in the targeted ribosomes (Fernández-Puentes and Vázquez 1977; Hausner et al. 1987; Brigotti et al. 1989; Miller and Bodley 1991; Nierhaus et al. 1992). In a more recent study, a single-molecule based analysis showed that cleavage of the SRL allowed initial binding and GTPase activation by EF-Tu ternary complex but blocked further progress to the accommodation step (Blanchard et al. 2004). The single-molecule study with EF-Tu motivated us to examine whether EF-G can also transiently interact with the SRL-cleaved ribosome and activate GTP hydrolysis.

Translocation catalyzed by EF-G consists of many sub-steps that are poorly understood (Wintermeyer et al. 2004; Frank et al. 2007; Pan et al. 2007; Spiegel et al. 2007). Binding of EF-G-GTP to the ribosome induces a ratchet-like rotation of the small subunit relative to the large subunit and stabilizes the P and A site tRNAs in the P/E and A/P hybrid states (Frank and Agrawal 2000; Spiegel et al. 2007). This is followed by GTP hydrolysis and conformational changes in the ribosome, called “unlocking”, which allow the rapid movement of the mRNA-tRNA complex (Katunin et al. 2002; Savelsberg et al. 2003). After translocation, the ribosome undergoes additional conformational changes called “relocking” and EF-G-GDP dissociates (Savelsbergh et al. 2003). Even though the ribosome undergoes extensive structural rearrangements, the SRL maintains its interaction with EF-G in the pre- and post-translocation state (Agrawal et al. 1999; Frank and Agrawal 2000; Stark et al. 2000; Connell et al. 2007). The SRL may, thus, play an important role in stabilizing the various transition states of EF-G during translocation. Interestingly, the SRL interacts intimately with the GTP-binding domain of EF-G implying a more direct role in catalyzing GTP hydrolysis (Connell et al. 2007; Taylor et al. 2007).

We show that cleavage of the SRL inhibits EF-G-dependent translocation, while sparsomycin-dependent translocation is not affected (Figures 4 and 5). These results are consistent with a previous report that EF-G-dependent translocation is inhibited, while spontaneous translocation is not (Hausner et al. 1987). Therefore, the intrinsic ability of the ribosome to translocate tRNAs is not inhibited by cleaving the SRL. Moreover, sparsomycin-dependent translocation also requires the tRNAs to be in the hybrid state suggesting that a SRL cleaved

ribosome can form this translation intermediate (Dorner et al. 2006). Therefore, changes produced in the SRL once cleaved do not appear to have long range effects, but mainly affect rapid translocation catalyzed by EF-G.

Our results show that cleavage of the SRL significantly reduces the binding of EF-G and inhibits its GTP hydrolysis activity (Figure 6). The strong defect in GTP hydrolysis corroborates the binding deficiency when GTP and not an analog is used. These data also allows us to discard the possibility of a further progress to the GTPase-activated state as is the case for EF-Tu.

The SRL interacts differently with EF-G and EF-Tu

In this study, binding of both elongation factors to the ribosome was analyzed and only EF-Tu can bind to the cleaved SRL (Figures 3 and 6), suggesting that they interact with the ribosome in a different conformation during the process of elongation. This is consistent with the single-molecule study that showed EF-Tu ternary complex binding to unmodified ribosomes and SRL cleaved ribosomes with equal efficiency (Blanchard et al. 2004). According to these authors, the SRL cleaved ribosomes stall at the tRNA accommodation step after ternary complex binding. In our case, much longer times of dipeptide reaction give a significant fraction of accommodated tRNA, suggesting that this is a very slow reaction in a SRL-cleaved ribosome. Thus, initial binding of the EF-Tu ternary complex to the ribosome is not significantly affected by cleavage of the SRL, but subsequent events in tRNA selection are inhibited. This is markedly different from the effects on EF-G, which requires an intact SRL for stably binding to the ribosome. In agreement with this observation, other kind of ribosome-inactivating proteins, the N-glycosidases, have been observed to differentially affect the function of elongation factors in a eukaryotic system by depurinating a specific base in the SRL (Xu and Liu 2000). Finally, the antibiotic thiostrepton weakens the interaction of EF-G with the ribosome by interacting with the GAC and L11 (Rodnina et al. 1999; Cameron et al. 2002; Seo et al. 2006; Harms et al. 2008). The defect observed with a cleaved SRL appears very similar to thiostrepton suggesting that a network of interactions between GAC, SRL, L11 and EF-G are required for the stability of EF-G on the ribosome and for translocation. In contrast, thiostrepton bound to the GAC does not affect the initial binding of EF-Tu ternary complex (Blanchard et al. 2004; González et al. 2007).

These differences in interaction of the SRL with EF-Tu and EF-G may support the idea that the ribosome switches between two different conformational states to discriminate between the elongation factors (Valle et al. 2003b). The sarcin/ricin loop is located at the surface of the ribosome but it is not a very mobile structure and it does not present dramatic conformational changes when elongation factors are bound. It has been proposed that an important determinant for binding one factor or the other is the distance between the SRL and the GAC (Sergiev et al. 2005). From our results we can conclude that EF-G binding is more dependent on the SRL conformation than the EF-Tu ternary complex. Binding of EF-Tu ternary complex may be stabilized by codon-anticodon interaction and other contacts with the ribosome, which may partially compensate for the loss of the SRL interaction.

Materials and Methods

Preparation of ribosomes, mRNAs, tRNAs, elongation factors and α-sarcin

Tightly coupled 70S ribosomes were isolated from *E. coli* MRE 600 cells in mid-log phase and purified by sucrose density gradient (Powers and Noller 1991). Phage T4 gene 32 mRNA was transcribed by T7 RNA polymerase from a linearized plasmid containing the appropriate insert. mRNA +9 was purchased from Dharmacon and labeled at the 3'-end with pyrene succinimide as described (Studer et al. 2003). EF-Tu, EF-Tu H84A and EF-G were overexpressed and purified as native forms using the IMPACT system (New England Biolabs) (Feinberg and Joseph 2006a). *E. coli* PheRS, MetRS and Methionyl-tRNA^{fMet} formyltransferase were produced as recombinant proteins with His-tag to facilitate their purification. Native *E. coli* tRNA^{fMet} and tRNA^{Phe} were purchased from Sigma and aminoacylated and purified as described before (Feinberg and Joseph 2001). *Aspergillus giganteus* natural α-sarcin was produced, isolated to homogeneity and characterized as described previously (Olson et al. 1965; Olson and Goerner 1965; Kao et al. 2001; Martínez-Ruiz et al. 2001).

Treatment with α-sarcin

Cleavage of 70S ribosomes by α-sarcin was assayed in four different buffers. Buffer A (polymix buffer): 20 mM Hepes-KOH (pH 7.6), 8 mM $MgCl_2$, 150 mM NH_4Cl, 4 mM 2-mercaptoethanol, 0.05 mM spermine and 2 mM spermidine (Bartetzko and Nierhaus 1988). Buffer B (A without polyamines): 20 mM Hepes-KOH (pH 7.6), 8 mM $MgCl_2$, 150 mM NH_4Cl, and 4 mM 2-mercaptoethanol. Buffer C (B with 6 mM EDTA): 20 mM Hepes-KOH (pH 7.6), 8 mM $MgCl_2$, 150 mM NH_4Cl, 4 mM 2-mercaptoethanol, and 6 mM EDTA. Buffer D (C with 8 mM EDTA): 20 mM Hepes-KOH (pH 7.6), 8 mM $MgCl_2$, 150 mM NH_4Cl, 4 mM 2-mercaptoethanol, and 8 mM EDTA. Different toxin concentrations and time of incubation were also tried. The highest efficiency of specific cleavage without unspecific degradation was obtained when 0.6 μM isolated 70S ribosomes were incubated with 0.3 μM α-sarcin during 30 min at 37ºC in buffer C. These conditions were used for cleaving the SRL in all subsequent experiments (α-sarcin-treated ribosomes). Intact ribosomes were also incubated without α-sarcin. A slightly increase in EDTA concentration from 6 mM (buffer C in Figure 1D) to 8 mM (buffer D in Figure 1D) made the ribosomes more susceptible to non-specific cleavage.

Most translation experiments involving prokaryotic ribosomes were usually performed in the standard polyamine buffer (buffer A). When the functionality of cleaved ribosomes was studied, standard buffer conditions were restored and activation of ribosomes was performed (incubation at 42ºC for 10 min and slow cool down to 37ºC followed by an additional incubation for another 10 min at 37ºC).

The integrity of α-sarcin cleaved ribosomes, before and after the functional assays was assessed by estimating their protein and rRNA content. The rRNA was phenol extracted and loaded onto a denaturing 2% agarose gel. The result was visualized by ethidium bromide staining. For protein content, ribosomes were separated from unbound proteins by filtration in 100 KDa MWCO

Microcon spin filters (Amicon). Bound and unbound fractions were analyzed by 17% SDS-PAGE and coomassie blue staining.

Primer extension analysis

The extent and site of α-sarcin cleavage was determined by primer extension. Reverse transcription using the primer 5'-ACCAGTGATGCGTCCACTCCG-3', complementary to the sequence 2634-2665 of *E. coli* 23S RNA (Figure 1B), and a mixture of dNTPs (-dATP) + ddATP, gave different products for an intact and a cleaved template. The uncleaved rRNA was transcribed up to the first uridine in the sequence due to the ddATP in the extension mixture (U2656). The extension with the α-sarcin cleaved template stopped at the cleavage site. The products of reverse transcription were separated in a 15% denaturing polyacrylamide gel and quantitated with a PhosphorImager (Molecular Dynamics).

In vitro translation assay

The overall activity of cleaved ribosomes was determined by an *in vitro* translation assay. Plasmid pRL-null (Promega) was modified by inserting a Shine-Dalgarno sequence (5'-**AAGGAGA**TATACAT**ATG**-3') upstream of the start codon of the *Renilla* luciferase gene. The plasmid was linearized with Bam HI and used as the template for coupled transcription and translation of the luciferase gene *in vitro*. The linearized plasmid (1µg) was mixed with 8 µl of synthesis mix (200 mM Hepes-KOH, pH 8.2, 7 mM DTT, 4 mM ATP, 3.3 mM of CTP, GTP and UTP, 0.1 M phosphoenolpyruvate, 7.5% PEG-6000, 0.13 mg/ml folinic acid, 240 U/ml pyruvate kinase, 0.14 M NH_4Ac, 0.28 M KAc), 4 µL of 100 mM $MgCl_2$, 4 µl of *E. coli* S100 extract and T7 RNA polymerase. This mix was incubated for 45 min at 37ºC to produce excess of mRNA for the assay. Then, 2 µl of 55 mM methionine, 6 µg of total tRNA from *E. coli* and 4 µl of 50 µM coelenterazine were added. The final volume of the reaction mixture was 55 µl. Activated ribosomes were prepared separately in standard buffer (6 pmols in 20 µl) and then combined with the reaction mixture and transferred to a 96-well plate. α-Sarcin treatment was performed either before the activation of the ribosomes or simultaneously with the translation reaction. The plate was incubated at 37°C in a plate reader (Genios, Tecan) and the synthesis of luciferase enzyme was monitored in real-time by measuring the luminescence every 90 seconds.

Peptidyl-transferase assay

The assay was performed essentially as described (Feinberg and Joseph 2006b). f[^{35}S]Met-tRNA^{fMet} was obtained by aminoacylation and formylation of tRNA^{fMet} in the presence of [^{35}S]Met. Initiation complexes (ribosomes with mRNA and initiator tRNA in P site) were formed by incubating activated 70S ribosomes (0.15 µM final concentration) with gene 32 mRNA fragment (0.3 µM final concentration) for 15 min at 37°C followed by the addition of f[^{35}S]Met-tRNA^{fMet} (0.25 µM final concentration) and further incubation for another 20 min. A-site was filled with Phe-tRNA^{Phe} or

ternary complex (0.4 µM final concentrations) for different times at room temperature. Ternary complex binding should be completed in a second time scale, whereas factor-independent binding is a very slow process (minutes). EF-Tu ternary complex was formed by incubating 9 µM EF-Tu with 1 mM GTP, 1 µL pyruvate kinase (10mg/mL) and 3 mM phosphoenolpyruvate for 30 min at 37ºC, then adding 3 µM Phe-tRNA^{Phe} and incubating again for 15 min at 37ºC. The excess of EF-Tu warranties that not free Phe-tRNA^{Phe} is present in the sample. The dipeptide formation (transfer of the fMet in P site to Phe-tRNA^{Phe} in A site) was initiated by Phe-tRNA^{Phe} or ternary complex addition and stopped at different times with 1/5 volumes of 1M KOH and placed on ice. The pH of the reaction mixture was neutralized and 1.5 µl aliquots were analyzed by electrophoretic TLC as described before (Feinberg and Joseph 2006b). The amount of f[^{35}S]Met-Phe and f[^{35}S]Met were quantitated using a PhosphorImager (Molecular Dynamics) and the fraction of dipeptide calculated per sample was normalized to the maximum formed with intact ribosomes in each experiment.

Translocation assays

Standard toeprinting assays for translocation were performed in standard polyamine buffer A as described before (Joseph and Noller 1998). Final concentrations were 0.15 µM ribosomes, 0.3 µM gene 32 mRNA hybridized with 5'-[^{32}P]-AL2 primer, 0.35 µM tRNA^{fMet} or fMet-tRNA^{fMet} for the P site and 0.5 µM of tRNA^{Phe} or Phe-tRNA^{Phe} for the A site. After the addition of EF-G•GTP (1.2 µM EF-G and 1 mM GTP final concentrations) aliquots were stopped after 10 min at room temperature by placing them on ice. The sparsomycin-induced translocation was performed according to Fredrick and Noller (Fredrick and Noller 2003). Ribosomes programmed with mRNA301 contained tRNA$_2^{Tyr}$ in the P site N-acetyl-Phe-tRNA^{Phe} in the A site. Translocation was induced by adding 0.5 mM sparsomycin (final concentration). For the time course analyses, aliquots were withdrawn at the various time points and mixed with viomycin (1 mM final concentration) to stop the reaction. Reverse transcription and gel analysis followed standard procedures (Joseph and Noller 1998). The gels were quantitated using a PhosphorImager (Molecular Dynamics).

Steady-state fluorescence measurements were also used to analyze translocation (Studer et al. 2003). Activated ribosomes (0.25 µM) were incubated with a 21 nucleotide pyrene-modified mRNA+9 (0.5 µM) for 10 min at 37ºC. Pre-translocation complexes were formed by adding fMet-tRNA^{fMet} (0.5 µM) and incubated for 20 min at 37ºC followed by Phe-tRNA^{Phe} (0.6 µM) for another 20 min. Post-translocation complexes were obtained by adding 10 µl of EF-G•GTP mix (0.8 µM and 1 mM respectively, final concentrations) to the pre-translocation complexes and incubating at 25ºC for 15 min. Samples were excited at 343 nm and the emission spectrum of pyrene from 360–420 nm was recorded at 25ºC in a photon-counting fluorometer (Fluoromax-P, JY Horiba). Translocation was followed by a decrease of fluorescence emission at 376 nm (Studer et al. 2003).

EF-G-dependent GTP hydrolysis

Activated ribosomes (0.2 µM final concentration) were mixed with EF-G (from 1 to 9 µM final concentration) and GTP (1mM final concentration with traces of [γ-^{32}P]-GTP, specific activity 25

Ci/mmol) at room temperature. Aliquots at different times were quenched with 5% SDS (1.25% final concentration). 1 µl samples were spotted on PEI cellulose TLC plates and developed in 0.5 M KH_2P0_4 (pH 3.5). The amount of $^{32}P_i$ formed was quantitated using a PhosphorImager (Molecular Dynamics). The initial velocities (as GTP hydrolyzed/ribosome/sec) were plotted versus each EF-G concentration and fitted to a Michaelis-Menten equation. The analysis of the data gave k_{cat}, K_M and k_{cat}/K_M kinetic constants of intact and 75% α-sarcin-cleaved ribosomes. The parameters for a population containing only α-sarcin-cleaved ribosomes were obtained according to Saarma et al. (1997), using the equations:

$$k_{catsar}/K_{Msar}=[(k_{catmix}/K_{Mmix})-(1-0.75)k_{catWT}/K_{MWT}]/0.75$$

$$K_{Msar}=0.75/[(1/K_{Mmix})-(1-0.75)/K_{MWT})]$$

Where, sar, mix and WT stand for pure cleaved ribosome population, cleaved ribosomes contaminated with 25% of intact ribosomes and intact ribosomes, respectively.

EF-G and EF-Tu-ternary complex binding to ribosomes

Binding of EF-G and EF-Tu ternary complex to the ribosome was analyzed by filtration (Wilson and Nechifor 2004). For EF-G binding, activated ribosomes (0.2 µM final concentration) were formed in standard polyamine buffer in 60 µl volume. Then, 20 µL of EF-G solution (minimum of 0.4 µM final concentration) with GTP or GDPNP (1 mM final concentration) were added to the reaction and incubated for 5 min at room temperature before filtering. For ternary complex binding, initiation complexes were formed in 60 µl final volume (0.2 µM ribosomes, 0.4 µM gene 32mRNA and 0.4 µM fMet-$tRNA^{fMet}$, final concentrations). EF-Tu (or EF-Tu H84A) ternary complex was formed by incubating 1.6 µM of elongation factor with 4 mM GTP (or GDPNP) for 45 min at 37ºC, then adding 2 µM Phe-$tRNA^{Phe}$ and incubating again for 15 min at 37ºC. 20 uL of ternary complex (0.4 µM final concentration) in the presence or absence of the antibiotic kirromycin (25 µM final concentration) were added and incubated for 5 min at room temperature. Unbound EF-G or ternary complex was removed using 100,000 MWCO Microcon spin filters (Amicon) and washing the samples twice with 300 µL of buffer containing the corresponding nucleotide and antibiotic. The washed samples were concentrated to 10 µl and analyzed by SDS-PAGE. The gels were scanned and the amount of factor bound to the ribosome was determined relative to the ribosomal protein S1 using ImageQuant (Molecular Dynamics).

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Resultados C

POSIBLES APLICACIONES CLÍNICAS DE LAS RIBOTOXINAS

La ribotoxina Asp f 1 es uno de los principales alérgenos de *Aspergillus fumigatus*, el hongo anemófilo patógeno más común. La parte más expuesta al disolvente de su horquilla β amino-terminal (residuos 7-22) está implicada en, al menos, un epítopo alergénico. Se puede considerar que la α-sarcina, producida por un hongo no patógeno, *A. giganteous*, es una variante natural de Asp f 1, pues ambas proteínas presentan una identidad de secuencia del 87% y una estructura tridimensional casi idéntica. Más de una cuarta parte de los 19 residuos que las diferencian se encuentran en esa horquilla β. Estudios previos mostraron una menor reactividad a IgE de los mutantes de deleción de Asp f 1 y α-sarcina, así como de la propia α-sarcina silvestre, aunque los epítopos IgG se conservaban, lo que convertía a las tres moléculas en buenos candidatos para la diagnosis y las terapias inmunomoduladoras en la hipersensibilidad a *Aspergillus*.

C1. Una variante de deleción del alérgeno Asp f 1 provoca una inflamación alérgica de las vías respiratorias atenuada en un modelo múrido de sensibilización.

Como se ha mencionado, la variante de deleción α-sarcina Δ(7-22) es un prometedor candidato para su uso en inmunoterapias de casos de hipersensibilidad a *Aspergillus*, pues carece de actividad citotóxica frente a células de rabdomiosarcoma humano y, además, presenta una reactividad muy reducida frente a las IgE anti Asp f 1. El primer paso a dar en este sentido es una evaluación *in vivo* de sus propiedades hipoalergénicas. Para ello, en este trabajo se estableció un modelo de ratón sensibilizado frente a Asp f 1, administrando de forma intraperitoneal e intranasal una mezcla de la proteína Asp f 1 recombinante purificada y una suspensión de un extracto de *Aspergillus fumigatus*. Los ratones sensibilizados presentaron niveles elevados de IgE en suero junto con lesiones histológicas en pulmones y fosas nasales. En cambio, cuando en las dosis intranasales se administró el mutante α-sarcina Δ(7-22) en vez de Asp f 1, estos síntomas se vieron muy reducidos, verificándose la menor alergenicidad del mutante de deleción.

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A deletion variant of the allergen Asp f 1 attenuates *Aspergillus fumigatus* induced allergic airway inflammation in a mouse model of sensitization.

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Short title: Asp f 1 allergen variant with diminished allergic response

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Keywords: *Aspergillus*, sarcin, ribotoxin, Asp f 1

Abstract

Background: *Aspergillus fumigatus* is the most prevalent airbone fungal pathogen, being the ribotoxin Asp f 1 one of its major allergens. α-Sarcin is a natural variant of Asp f 1 produced by the non-pathogenic fungus *Aspergillus giganteus*. Both proteins show sequence identities of 87% and almost identical three-dimensional structures. The α-sarcin Δ(7-22) deletion variant was shown to be much less cytotoxic against human rhabdomyosarcoma cells and indeed it displayed a 50% reduction of Asp f 1-IgE reactivity.

Objectives: To establish a murine model of IgE sensitization to Asp f 1 and to use it to test the immunologic response elicited by the α-sarcin Δ(7-22) mutant.

Methods: BALB/c mice were sensitized with mixtures of recombinant wild type Asp f 1 and/or an *Aspergillus fumigatus* extract and challenged with Asp f 1 or α-sarcin Δ(7-22). Mice sera were collected for subsequent measurement of Ig levels and histological analysis of nostrils and lungs was performed.

Results: Development of a murine model of sensitization to Asp f 1 was only possible when the purified protein Asp f 1 was administered together with an extract of *A. fumigatus*. The model was characterized by elevated levels of total IgE in serum and histological lesions in lung and nostrils. These symptoms were greatly reduced when the deletion variant was the protein administered.

Conclusions: A mouse model of *A. fumigatus* sensitization has been established and used to show the decreased allergenicity of α-sarcin Δ(7-22) which appears as a suitable candidate to test for future immunotherapy.

Introduction

Aspergillus fumigatus is an exceptional fungus among microorganisms in being both a primary and opportunistic pathogen as well as an important source of allergens [1]. Its ubiquitous spores are continuously inhaled by humans leading to an almost constant exposure of the respiratory tract and, therefore, to the possibility of being colonized [2]. This inhalation has adverse effects only very rarely as the conidia are usually eliminated efficiently by the innate immune system. Nevertheless, immunosuppressive therapies are changing significantly this scenario leading to an increase of the onset of pathologic events involving *A. fumigatus* infections. Perhaps for all these reasons, this microorganism is now the most prevalent airborne fungal pathogen [2] and the etiological agent identified in 80% of *Aspergillus*-related diseases, including hypersensitivity pneumonitis, allergic rhinitis, IgE-mediated asthma, and the severe allergic bronchopulmonary aspergillosis (ABPA), as well as different invasive infections in immunocompromised patients [1]. ABPA is characterized by the presence of specific IgE against a number of *A. fumigatus* proteins, enhanced peripheral blood and lung eosinophilia, mild bronchospasm and airway hyperreactivity to inhaled allergens [1,3].

Over the past few years, a number of allergens have been identified and characterized from *A. fumigatus*. Asp f 1, a protein belonging to the ribotoxins family, is one of its major allergens, showing prevalence higher than 80% [4,5]. Asp f 1-specific IgE antibodies are generally found in sera from patients sensitized to *Aspergillus*, but particularly in ABPA patients [1,5-6].

α-Sarcin from the non-pathogenic fungus *Aspergillus giganteus* is the best characterized member of the fungal ribotoxins family, a group of highly specific secreted ribonucleases [7]. Asp f 1 belongs to the same family and shows 87% amino acid sequence identity with α-sarcin [4] as well as almost identical three-dimensional structures [5, Yang and Moffat, 1996, Campos-Olivas et al., 2001]. In this regard, both proteins can be considered as natural variants from the same toxins family. Their toxicity comes first from their ability to reach the cytosol via receptor-independent endocytosis [8]. Then, once inside the host cell, ribotoxins inhibit protein biosynthesis by inactivating the ribosomes, leading to cell death [7]. Based on mutational studies of α-sarcin, the NH_2-terminal β-hairpin has been shown to play a key role both in the cytotoxic effect of ribotoxins as well as in IgE mediated

responses [5,9]. According to these data, the deletion mutant α-sarcin Δ(7-22), where this β-hairpin had been eliminated [9], showed a much lower cytotoxicity and a 50% reduction of IgE reactivity. Interestingly, the mutant still maintained the wild-type three-dimensional structure [García-Mayoral et al., 2005] and its prevalence in sera from ABPA patients [5].

Currently, immunotherapy of *A. fumigatus* allergy is performed with allergenic extracts containing up to 200 different allergens, which are difficult to standardize [10]. In addition, the risk of anaphylactic side reactions during the treatment cannot be ruled out. These problems could be overcome with the use of recombinant allergens with reduced IgE-binding capacity [11-13]. In this regard, the deletion variant of ribotoxins described above is a promising molecule for use in immunomodulating therapies for *Aspergillus* hypersensitivity. Taking into account its high sequence and three-dimensional structure identities, altogether with its reduced IgE binding capacity, α-sarcin Δ(7-22) can be also considered as an hypoallergenic variant of Asp f 1. However, the *in vivo* evaluation of such hypoallergenicity is still required to assess this possibility. Therefore, the primary aim of this study was to establish a murine model of IgE sensitization to the recombinant major allergen of *Aspergillus fumigatus* Asp f 1 and then to use it to test the immunologic response elicited by this deletion variant [5,9].

Materials and methods

Mice

Female, 6-8 week old BALB/c mice were obtained from Harlan Interfauna Ibérica SA (Barcelona, Spain). Animals were maintained at the Animal Care Services of the Faculty of Biology (Universidad Complutense, Madrid, Spain), according to the local guidelines for animal care. The studies were approved by the Animal Experimentation Ethics Committee of the Complutense University.

Protein production and purification

E. coli BL21 (DE3) cells cotransformed with a thioredoxin-producing plasmid (pT-Trx) and the corresponding Asp f 1 or the deletion mutant α-sarcin Δ(7-22) plasmids were used to produce the proteins, as previously described [14-16]. Protein purification included ion exchange and molecular size exclusion chromatographies [17]. SDS-PAGE of proteins, protein hydrolysis, amino acid analysis, and circular dichroism spectra were performed according to standard procedures [6,14,17]. Western blots were performed as described before [5] using rabbit sera raised against wild-type α-sarcin or Asp f 1. Homogeneity of the protein samples used in this study was assessed according to all these previous criteria, as describe before.

Aspergillus fumigatus extract preparation

Spores and mycelia inactivated by gamma radiation (Allergon AB, Ängelholm, Sweden) were resuspended in PBS 1mg/ml (w/v) and this suspension was shaken for 2 hours at 250 rpm and 4ºC. This suspension is referred as the "Af extract" through the text.

Induction of experimental allergy (sensitization and challenge)

Mice (n=5) were sensitized by 2 intraperitoneal (i.p.) injections with 1 µg of Asp f 1 and 10 µl of the Af extract adsorbed to 2 mg $Al(OH)_3$ in 150 µl of PBS in 7 day-intervals. After 7 days mice were challenged by intranasal (i.n.) administration of different amounts of Asp f 1 or the mutant α-sarcin Δ(7-22) in 50 µl PBS on 3 consecutive days under anaesthesia (Fig.1, table 1). Seven days after the last i.n. challenge, blood samples were collected from the retroorbital plexus (terminal bleeding) and tissues were removed for histological examination.

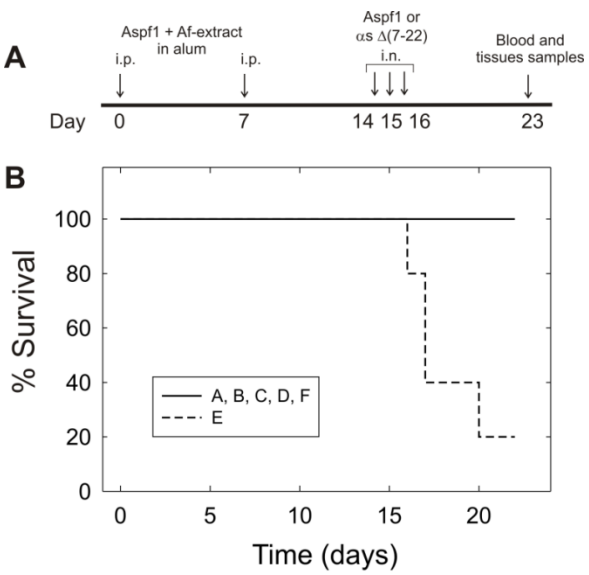

Fig. 1. – (A) Experimental protocol. Mice were sensitized by intraperitoneal (i.p.) Asp f 1 (1µg) and Af extract (10 µl) on $Al(OH)_3$ followed by intranasal (i.n.) challenge with Asp f 1 (1, 5 or 10 µg) or α-sarcin Δ(7-22) (1µg) on three consecutive days. After one week, blood and serum tissues samples were collected. (B) Survival of mice treated according to these protocols, expressed as percentage of mice surviving as a function of time of treatment. A to E are the different mice group (see Table 1).

Table 1. - Different doses of Asp f 1 and Af extract used in the final sensitization protocol. The intranasal administrations of Asp f 1 were of 1, 5, or 10 µg.

Mice group	Intraperitoneal injection	Intranasal challenge
A	None	None
B	10 µl Af extract + 1 µg Asp f 1	PBS
C		1 µg Asp f 1
D		5 µg Asp f 1
E		10 µg Asp f 1
F		1 µg α-sarcin Δ(7-22)

Determination of specific allergen IgG1, IgG2a and total IgE in serum

Antibodies (Ab) binding was measured by ELISA as previously described [18]. In order to quantitate Asp f 1 specific antibodies, serum samples were diluted 1:25000 for IgG_1, 1:400 for IgG_{2a}, and 1:5 for IgE. Antibody levels were expressed as optical density (OD) values at 492 nm. Total IgE (diluted 1:40) levels in serum were measured by sandwich ELISA using the OptEIA mouse IgE set (PharMingen, San Diego, CA, USA) according to the manufacturer's instructions.

Histopathology

Nostrils and lung samples were fixed in 10% buffered formalin, routinely processed and paraffin-embedded. Sections of 3 µm were stained with haematoxylin and eosin (H&E) to examine the general morphology and cellular infiltration and Periodic acid-Schiff (PAS) technique to observe the mucus production. Sections were examined under light microscope by pathologist blinded to the protocol design. The intensity and severity of lung and nostrils affections were assessed on a semiquantitative score ranging from 0 to 4.

Results

Sensitization protocol

In order to establish a murine model of allergic sensitization to recombinant Asp f 1, different approaches were tried. The success of each protocol was evaluated by means of measuring total and specific antibody levels, including IgE, IgG_1 and IgG_{2a}, as well as by assessing clinical manifestations such as histopathological alterations, pilar erecti and weight-loss. The final strategy tried was based on the intraperitoneal co-administration of recombinant Asp f 1 and Af extract in alum, followed by i.n. challenge with different doses of recombinant Asp f 1 (C, D, and E groups; Table 1). Group A (non-treated mice) and group B (mice challenged with PBS) represent controls. Mice in group E systematically died after the third i.n. challenge (Fig.1). Consequently, it was not further studied. Groups C and D did experience adverse effects, as evidenced by weight loss and pilar erecti, but fully recovered once the Asp f 1 challenge was interrupted.

Antibody response

Intraperitoneal injection of the Asp f 1–Af extract mixture was sufficient to induce a seven-fold increase in the total IgE serum levels when comparing to group A (Fig. 2). This response was further strengthened after i.n. challenge with 5 µg of Asp f 1 (Fig. 2). No significant differences were observed between groups B and C. Asp f 1-specific IgE and IgG_1 antibody levels were not detected in the sera of any of the immunized mice within the limits of ELISA and Western blot.

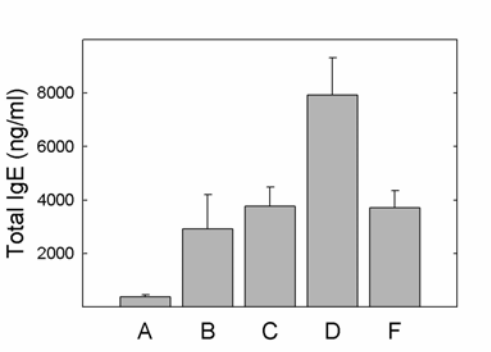

Fig. 2. - Total serum IgE levels in the different groups of sensitized mice determined by ELISA. Bars represent group means ± standard error (SEM) of 5 mice per group.

Lung and nostril histology

Lung tissues were obtained from each experimental group, except for group E, seven days following the last i.n. challenge to assess the effect of the sensitization protocol on airway inflammation. Histological examination revealed that Asp f 1 challenge promoted severe perivascular and peribronchial infiltrations of inflammatory cells -lymphocytes, neutrophils and occasional eosinophils and macrophages- in all lobes of lungs, in comparison with naïve mice. These effects were even more evident in mice of group D where a marked reepithelization and fibrosis, characterized by an enrichment of collagen fibers, were also observed in all over the parenchyma (Fig. 3).

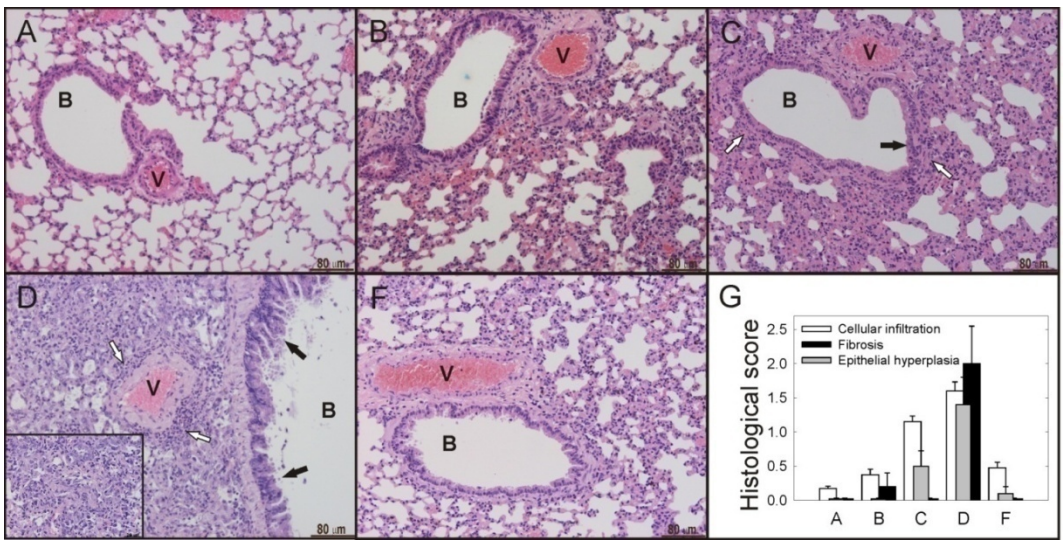

Fig. 3. – (A-F) Representative photomicrograph H&E stained of lung section from mice in groups A, B, C, D, and F. Histological examination revealed numerous zones of inflammation (white arrows) and epithelial hyperplasia (black arrows) in lungs from C and D animals in comparison to control mice (groups A and B). B, bronchium; V, Blood vessel. Fibrosis was especially noteworthy in group D (See the inset photomicrograph in panel D). (G) Histological score for lung inflammation. Data are the mean ± SEM of 5 mice per group.

Examination of nostril tissues revealed that they were also affected by Asp f 1 sensitization (Fig. 4). A mixed infiltrate of cells, especially lymphocytes and neutrophils, were observed into the nostrils compared to naïve mice. Again, the most severe effects were obtained in group D. Mucus hypersecretion in the nostrils cavities was a notable histopathological feature of the Asp f 1 challenged mice.

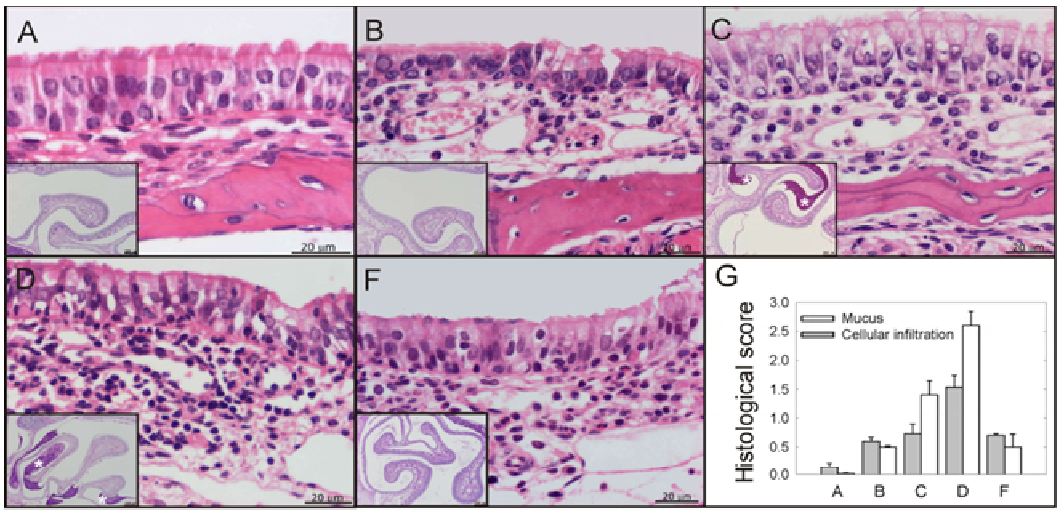

Fig. 4. – (A-F) Representative photomicrograph of H&E stains of nostrils sections from mice in groups A, B, C, D and F showing the cellular infiltration. The insets are photomicrograph PAS stains of a wider field where the mucus plugs are marked with asterisks. (G) Histological score of inflammation and mucus secretion. Data are the mean ± SEM of 5 mice per group.

Effect of a deletion mutant with diminished IgE response

The Δ(7-22) deletion mutant of α-sarcin was also included in this study with the final goal of evaluating its effect on the onset of the allergic sensitization. Thus, mice of group F were intranasally challenged with 1 µg of Δ(7-22) α-sarcin, the amount of recombinant Asp f 1 used for animals in group C. No significant effects on total IgE were observed in the mutant challenged mice compare to group C (Fig. 2). Nevertheless, i.n. administration of the mutant decreased the perivascular and peribronchial cellular infiltration and inhibited epithelial cell hyperplasia in lung (Fig. 3). With respect to nostrils, the use of the mutant protein failed to induce a lower cellular inflammatory response, but reduced notably mucus release (Fig. 4).

Discussion

One of the alternative approaches to conventional immunotherapy for allergy is based on the use of hypoallergenic derivatives as vaccines [11,19]. Before clinical application, *in vitro* and *in vivo* evaluation of hypoallergens is required to identify the best candidates. In the present study, the *in vivo* allergenicity of a potential hypoallergenic variant of Asp f 1, α-sarcin Δ(7-22) mutant, has been investigated in a mouse model of *A. fumigatus* sensitization in comparison to the recombinant wild-type protein. Previously, the murine model of sensitization to this fungus has been established.

Murine models mimicking clinical events are useful tools as preclinical test systems for new prophylactic and therapeutic approaches against allergy, as well as for studying the immunological events involved in Type I sensitization. The majority of animal models of *A. fumigatus* sensitization have been achieved in mice by i.n. administration of crude extracts of the fungus or with intact organisms, particularly the conidia [20-22]. Although it has been described that potent sensitization occurs in absence of exogenous adjuvants [23], we were not able to sensitize mice to the commercially available extract used in this study. This could be due to the no detectable levels of Asp f 1 in it. On the basis of previous studies [20-22,24-26], BALB/c mice were then sensitized by i.p. administration of Asp f 1/Af extract in $Al(OH)_3$ followed by i.n. challenge. The allergic state was characterized by the induction of high levels of total IgE in serum, which were further enhanced after i.n. challenge, as well as histological lesions in the lung and nostrils. However, neither Asp f 1-specific IgE, IgG_1 nor IgG_{2a} antibodies were detected. This is in agreement with the studies of Svirshchevskaya *et al.* [27] who observed that immunization of animals with *A. fumigatus* extract induced an increased level of total serum IgE, but not specific IgE/IgG antibodies. Lung lesions typically consisted of perivascular and peribronchial infiltrates of inflammatory cells, emigration of some eosinophils into the lumen and in severe cases epithelial hyperplasia. Accumulation of collagen was frequently noted at the parenchymal area. In addition, the strong inflammation in the lungs induced by *A. fumigatus* antigens persisted over seven days after the last i.n. challenge. Similar results have been also obtained by other groups [28-30]. The histological study was further extended to the nostrils of the animals and the obtained results were compatible with the induction of an allergic response to *A. fumigatus* antigens. Overall, the results suggest that the allergic response observed in the present murine model is not just the typical IgE-dependent response but rather the result of a much more complex interaction between the host immune system and *A. fumigatus* antigens, including ribotoxins. The specific roles of the individual allergens are still to be clearly determined. These results suggest that the allergic state induced by Asp f 1/Af extract could result from the cumulative effects of various toxins, esterases and proteases present in the extract that can act as adjuvants, perhaps by inducing epithelial damage and allowing normally antigens to bypass the mucosal barrier [22,26,31]. In addition, it has been shown that proteases preferentially induce Th2 responses, suggesting that they could be skewing the response to *A. fumigatus* antigens to a more allergic phenotype [32, Lamhamedi-Cherradi et al., 2008].

Finally, the most encouraging results presented in this work are probably those obtained with the hypoallergenic derivative of Asp 1. Few studies have analyzed the ability of hypoallergens to

induce an IgE response in an animal model [33-3]. Challenge of mice with the α-sarcin Δ(7-22) mutant resulted in substantially decrease in lung and nostril lesions when compared to that induced by wild type Asp f 1, but total IgE levels remained unaffected. Our data demonstrated that this mouse model of *A. fumigatus* sensitization is a suitable system for the *in vivo* testing of mutant allergens. Moreover, these data confirmed the low toxicity and the hypoallergenic character suggested previously for the deletion mutant [5]. Obviously, toxicity at higher doses cannot be ruled out and therefore must be explored further in the near future. However, the good correlation of *in vivo* and *in vitro* experiments allows us to consider the α-sarcin Δ(7-22) mutant as a reasonable vaccine candidate.

In summary, a mouse model of *A. fumigatus* sensitization has been established. Despite of its limitations, this *in vivo* model has provided valuable information for preclinical testing of recombinant allergen and its derivatives. The decreased allergenic activity of α-sarcin Δ(7-22), in terms of a reduced airways inflammatory response and the previously described [5] lower human IgE binding activity, identified this molecule as suitable candidate for immunotherapy, although further studies are required to establish its efficiency and safety for treatment of allergic patients.

Acknowledgements

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C2. *Lactococcus lactis* como vehículo para la expresión heteróloga de variantes de ribotoxinas fúngicas con afinidad reducida por IgE.

Lactococcus lactis es una bacteria Gram positiva, no patogénica, no invasiva, ni colonizante, considerada GRAS (*generally regarded as safe*), ideal como vehículo para la administración de fármacos o vacunas en nuestro tracto digestivo. Con esta idea en mente, se obtuvieron cepas de *L. lactis* capaces de producir y secretar el alérgeno Asp f 1 y las tres variantes con afinidad reducida por IgE: la α-sarcina silvestre y ambos mutantes de deleción. Además, se incluyó un mutante de la α-sarcina sin actividad ribonucleolítica y por tanto no citotóxico: α-sarcina H137Q. Con un medio de cultivo tamponado, las proteínas se secretan al medio extracelular en su forma nativa, pues presentan actividad ribonucleolítica, salvo el mutante catalítico, obviamente.

Como una primera prueba, se administró intragástricamente la cepa productora de α-sarcina silvestre a ratones sanos durante un periodo de 14 días, resultando inocua. En resumen, los resultados que se presentan se discuten en términos de su potencial aplicación como vehículo de distribución oral de variantes hipoalergénicas así como de punto de partida con el que enfocar el diseño de estrategias para lograr una forma segura de distribuir estas proteínas como agentes antitumorales.

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Lactococcus lactis as a vehicle for the heterologous expression of fungal ribotoxin variants with reduced IgE-binding affinity

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Abstract

Fungal ribotoxins are a family of extracellular ribonucleases which inhibit protein biosynthesis by inactivating the ribosomes. This inactivation results in the induction of cell death by apoptosis. Ribotoxins show antitumoral properties based on their ability to cross the membrane of some transformed cells. Unfortunately, they also show an unspecific cytotoxicity which has greatly impaired their potential clinical uses. α-Sarcin, produced by *Aspergillus giganteus*, is the best-characterized ribotoxin. Asp f 1, another ribotoxin produced by *A. fumigatus,* is indeed one of its major allergens. In this work, the *Lactococcus lactis* MG1363 strain has been engineered to produce and secrete not only wild-type Asp f 1 and α-sarcin but also three different mutants with reduced cytotoxicity and/or IgE-binding affinity. The proteins were secreted in native and active form when the extracellular medium employed was buffered at pH values around 8.0. Strains producing the wild-type natural α-sarcin were proved to be innocuous when administered intragastrically to mice for a period of 14 days. Overall, the results presented are discussed in terms of its potential application as a vehicle of oral delivery of hypoallergenic variants as well as a starting point to approach the design of strategies to accomplish the safe delivery of these proteins as antitumoral agents.

Keywords: α-Sarcin; Asp f 1; Ribonuclease; Probiotic

1. Introduction

Aspergillus species are responsible for several human lung pathologies, including different allergic inhalant diseases (Kurup et al., 2002), allergic bronchopulmonary aspergillosis (ABPA) being the most severe form among them. *Aspergillus fumigatus* is usually the mold involved in most of those diseases, ABPA included, because this fungus, with small spores, optimally grows at 37 °C, a temperature that is prohibitive for most of the other environmental ubiquitous fungi. Thus, it can colonize the respiratory tract of the host leading to the onset of pathological events (Banerjee and Kurup, 2003).

Ribotoxins are a group of secreted fungal ribonucleases, best represented by α-sarcin (Lacadena et al., 2007), whose toxicity comes from their ability to reach the cytosol via endocytosis without establishing any receptor interaction (Olmo et al., 2001). They inhibit protein biosynthesis by inactivating the ribosomes (Schindler and Davies, 1977; Kao et al., 2001) which results in induction of cell death by apoptosis (Olmo et al., 2001). This ribosome inactivation is achieved by cleaving a unique phosphodiester bond at the so-called sarcin–ricin loop (SRL) of the largest rRNA (Endo and Wool, 1982; Correll et al., 1999). Ribotoxins were discovered during a screening program of the Michigan Department of Health searching for antibiotics and antitumor agents (Olson et al., 1965). Unfortunately, further studies revealed an unspecific cytotoxicity of these proteins, which limited their potential clinical uses (Roga et al., 1971).

All ribotoxins show a high degree of sequence identity with most of their differences appearing on exposed regions, such as their NH_2-terminal β-hairpin (α-sarcin residues 1–26), a domain

Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; SRL, sarcin–ricin loop; GRAS, "Generally Regarded As Safe"; PCR, polymerase chain reaction; H&E, haematoxylin and eosin.

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which can be considered as two consecutive minor β-sheets connected by a hinge region. The second of them (residues 7–22) juts out as a solvent-exposed protuberance and is one of the protein regions with highest conformational flexibility (Pérez-Cañadillas et al., 2000, 2002). Deletion of this β-sheet results in a Δ(7–22) mutant that shows no significant conformational differences except for the deleted region (García-Mayoral et al., 2004) but has lost its ability to specifically recognize the ribosome and is much less cytotoxic (García-Ortega et al., 2002).

Asp f 1, the ribotoxin produced by *A. fumigatus*, is also a major and one of its best-characterized allergens (Moser et al., 1992). There is a significant prevalence of Asp f 1-specific IgE antibodies in sera from patients sensitized to *Aspergillus*. Particularly in ABPA, the detection of these antibodies seems to be a promising approach for its otherwise difficult diagnosis (Kao et al., 2001; Greenberger, 2002; Banerjee and Kurup, 2003; García-Ortega et al., 2005). Asp f 1 differs from α-sarcin in only 19 (87% sequence identity) out of 150 residues. Five of these differences are precisely located at the NH_2-terminal β-hairpin. Structural and immunogenic studies of Asp f 1, α-sarcin, and two variants where this β-hairpin had been deleted [Asp f 1 Δ(7–22) and α-sarcin Δ(7–22)] (García-Ortega et al., 2005) showed that the deleted portion is involved in at least one allergenic epitope (García-Mayoral et al., 2004; García-Ortega et al., 2005). In spite of their decreased IgE reactivity, the prevalence of the two deleted proteins among the sera of patients remained essentially unaffected while they still retained most of the IgG epitopes (García-Ortega et al., 2005). It was then concluded that these ribotoxins' variants might be suitable for use in immunomodulating therapies and diagnosis of *Aspergillus* hypersensitivity.

The digestive tract is inhabited by commensal flora, whose correct settlement has been shown to be very important for human health. In fact, some pathological states can be improved just by means of administration of certain live bacteria, the so-called probiotics (Hooper and Gordon, 2001; Schiffrin and Blum, 2001). In the last years, the setting-up of genetic engineering techniques has made possible the genetic modification of commensal bacteria in order to obtain "biodrugs", i.e., strains of bacteria capable of *in vivo* producing drugs, antimicrobial agents, or vaccines (Hooper and Gordon, 2001; Blanquet et al., 2001). One of the advantages of these "biodrugs" is the specific delivery of the therapeutic agent to their target.

Lactococcus lactis is a non-pathogenic, non-invasive, no colonizing Gram-positive bacterium, mainly used to produce fermented foods. This lactic acid bacterium holds "Generally Regarded As Safe" (GRAS) status and hence is a suitable candidate to be used as one of those "biodrugs". For example, it has been proven useful in producing IL-10 for the treatment of inflammatory bowel disease in mice (Steidler et al., 2000). In this work, the extracellular production by *L. lactis* of the above α-sarcin and Asp f 1 deletion variants is presented. Both wild-type proteins were also included in the study, as well as a properly folded, but catalytically inactive α-sarcin H137Q mutant, as a control (Lacadena et al., 1995; García-Ortega et al., 2002, 2005). The possibility of using this strategy as a potential immunomodulating therapeutic approach is discussed.

2. Materials and methods

2.1. Materials

All reagents were molecular biology grade. Restriction endonucleases and DNA modifying and synthesizing enzymes were purchased from Roche (Indianapolis, IN), New England Biolabs (Beverly, MA), or Promega (Madison, WI). Oligonucleotides were purchased from Sigma-Genosys (Cambridge, UK). PerkinElmer (Wellesley, MA) GeneAmpPCRSystem 2400 thermal cycler was used for the polymerase chain reaction (PCR)-based amplifications. DNA sequencing was performed at the facility of the Universidad Complutense (Madrid, Spain).

2.2. Media

L. lactis strain MG1363 (Table 1) was routinely grown at 30 °C in M17 (Difco) containing 0.5% (w/v) glucose (GM17) under static conditions, as described (Gil et al., 2001), adding erythromycin (5 μg/ml) when needed. In order to prepare electrocompetent cells, they were grown in GM17 medium but supplemented with 23.8 mg/ml L-threonine, and 1 mM $MgSO_4$ (GM17GT). Protein production was initially assayed in a medium (GM9) containing 0.042 M Na_2HPO_4, 0.02 M KH_2PO_4, 0.01 M NH_4Cl, 8.5 mM NaCl, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 5 g/l peptone, and 5 μg/ml erythromycin. Final production of the proteins was accomplished in 0.2 M potassium phosphate buffered GM9 (PM) adjusted at different pH values between 6.0 and 8.5.

2.3. Cloning procedures

Cloning procedures and DNA manipulations were carried out according to standard methods (Lacadena et al., 1994; Maassen, 1999; Schotte et al., 2000; Sambrook and Russell, 2001). Suitable deoxyoligonucleotides were used as primers for PCR amplification, using a series of plasmids constructed before as templates (Lacadena et al., 1994, 1995; García-Ortega et al., 2002, 2005), containing the cDNA corresponding to the five proteins studied: Asp f 1, α-sarcin, Asp f 1 Δ(7–22), and α-sarcins Δ(7–22) and H137Q. These amplified DNA fragments were flanked by NgoMIV and BamHI restriction sites that were used to clone the proteins into the corresponding cloning sites of the plasmid pT1NX, in frame with the secretion signal leader of the lactococcal *usp*45 gene placed under the control of the constitutive promoter P1 (Steidler et al., 1995, 2000). The vectors thus obtained (Table 1) were used to electroporate electrocompetent MG1363 *L. lactis* cells.

2.4. L. lactis electroporation

In order to prepare electrocompetent MG1363 *L. lactis* cells, the corresponding strain was grown overnight at 30 °C in 5 ml of GM17GT medium. Next day, this culture was diluted with fresh medium to a final volume of 25 ml and an OD_{600} of 0.1 and further incubated until reaching a value of 0.32. Cells were then harvested by centrifugation at 7500 × *g* for 15 min. The cel-

Table 1
Strain and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Reference
L. lactis MG1363	Plasmid-free derivative of NCDO712	Laboratory stock
pT1NX	*L. lactis* vector; erythromycin resistance; P1 promoter and *usp*45 secretion signal	Schotte et al. (2000)
pT1αsarcin	pT1NX with cloned *α-sarcin* cDNA	This work
pT1αsarcinH137Q	pT1NX with cloned *α-sarcin* H137Q variant	This work
pT1αsarcin7–22	pT1NX with cloned *α-sarcin* Δ(7–22) deletion variant	This work
pT1aspf	pT1NX with cloned *aspf1* cDNA	This work
pT1aspf 7–22	pT1NX with cloned *aspf1* Δ(7–22) deletion variant	This work

lular pellet was maintained in ice and washed twice with 2 ml of distilled water, once with 1 ml of 50 mM EDTA, and once with 1 ml of 0.3 M sucrose. Finally, washed cells were gently resuspended in 0.2 ml of 0.3 M sucrose and immediately used for electroporation. DNA ligation mixtures, or plasmids, used for electroporation were previously phenolized and ethanol precipitated in the presence of 50 μg/ml glycogen and redissolved in 10 μl of water. Electroporation was performed in a BioRad Gene Pulser apparatus at 2500 V, 200 Ω, and 25 μF, using 0.2 cm cuvettes and 2–3 μl (1 μg of DNA) of either the ligation mixtures or plasmids. Electroporated cells were quickly resuspended in 5 ml of GM17 containing 1% (w/v) sucrose and 1 mM $MgSO_4$ and incubated for 2 h at 30 °C. Selection of the proper colonies was then made for erythromycin (5 μg/ml) resistance on GM17 agar plates containing 1% (w/v) sucrose and 1 mM $MgSO_4$, after incubation at 30 °C.

2.5. Plasmids purification and analysis

Plasmids were purified essentially as described before (O'Sullivan and Klaenhammer, 1993; Maassen, 1999) by a method that combines enzymatic breakdown of the cell wall and purification of the plasmid by commercially available DNA-binding columns. With this purpose, the cells corresponding to 10 ml of a 30 °C GM17 erythromycin (5 μg/ml) containing overnight culture were pelleted, resuspended in 200 μl of 10 mM HCl–Tris, pH 8.0, supplemented with 20% (w/v) sucrose, 10 mM EDTA, 50 mM NaCl, and 15 mg/ml lysozyme, and further incubated for 15 min at 37 °C. This solution was then thoroughly but gently mixed with 200 μl of 0.2 M NaOH, containing 1% (w/v) SDS to induce the cell lysis. Cell lysates were subjected to fractionation using the standard procedure and reagents from GenElute™ Plasmid Miniprep Kit (Sigma–Aldrich). Isolated plasmids were analyzed by electrophoretic restriction maps and DNA sequencing.

2.6. Protein production

Individual *L. lactis* colonies harboring each one of the five pT1NX-derived plasmids (Table 1) containing the different ribotoxins' cDNA were cultured overnight at 30 °C in GM17 supplemented with 5 μg/ml erythromycin. Next day, 500 μl of these saturated cultures were used to inoculate 50 ml of the same medium and further grown at 30 °C to an OD_{600} of 0.3. These cultures were centrifuged at 5000 × *g* for 10 min at room temperature and the supernatant was discarded. The cellular pellet was gently resuspended in 50 ml of either GM9 or buffered PM media and incubated for five additional hours at 30 °C. These cultures were then processed as described in the following section.

2.7. Protein analysis and detection

L. lactis MG1363 grown for 5 h in GM9 or buffered PM media were centrifuged at 15,000 × *g* for 15 min at room temperature and the supernatants were then 20-fold concentrated using Microcon YM-10 centrifugal filter devices, for detecting the proteins by Western blot, or 250-fold using Centiprep YM-10 centrifugal filter devices as well, for detecting them by staining with Coomassie blue or zymogram. These samples were analyzed on 0.1% (w/v) SDS-PAGE performed in 15% (w/v) polyacrylamide gels (Laemmli, 1970). Gels containing the proteins thus fractionated were stained with Coomassie brilliant blue R-250 or blotted onto Immobilon (Millipore) membranes by semidry blotting for 1 h at 0.48 mA/cm^2. Immunodetection on these membranes was achieved as described (Lacadena et al., 1994; Villalba et al., 1994; García-Ortega et al., 2005) employing rabbit polyclonal antibodies raised against wild-type α-sarcin (Lacadena et al., 1994) or Asp f 1 (García-Ortega et al., 2005) and a secondary antibody horseradish peroxidase-labeled goat anti-rabbit IgG. The peroxidase reaction was developed using the ECL Western blotting reagent (Amersham Pharmacia Biotech) as described (Villalba et al., 1994; Barral et al., 2004). The ribonucleolytic activity of the proteins was detected by using a zymogram assay against poly(A). This assay was performed at pH 7.0 in 15% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS and 0.3 mg/ml of the homopolynucleotide. In these zymograms, proteins exhibiting ribonuclease activity appear as colorless bands after appropriate distaining (Lacadena et al., 1999; Kao et al., 2001; García-Ortega et al., 2002).

2.8. L. lactis oral administration to mice

Female, 6 weeks old, BALB/c mice were obtained from Harlan Interfauna Ibérica S.A. (Barcelona, Spain). Animals were maintained at the Animal Care Services of the Faculty of Biology (UCM, Madrid, Spain), according to the local guidelines for animal care. The studies performed were approved by the Animal Experimentation Ethics Committee of the Complutense University.

Mice ($n = 6$) were inoculated daily via intragastric gauge with 2×10^7 *L. lactis* cells suspended in 200 μl of bicarbonate solution during 14 consecutive days. Cells containing the α-sarcin producing pT1α-sarcin plasmid or the pT1NX vector were used. Simultaneously a control group of six mice remained untreated. Two weeks after last inoculation the animals were sacrificed and gut histology was analyzed for injury. Intestine samples from all animals were fixed with formalin, routinely processed and paraffin-embedded. Sections of 3 μm were stained with haematoxylin and eosin (H&E) technique.

These gut sections were examined under light microscope by the mice pathologist blinded to the protocol design.

For optimal α-sarcin production, the saturated cultures employed had been grown for 16 h in GM17 containing 5 μg/ml erythromycin and then were diluted 25-fold in buffered PM medium and further grown for three more hours at 30 °C. Then, the OD_{600} of these cultures was measured and they were diluted with the same medium in order to have the required 2×10^7 bacteria in 200 μl.

3. Results

3.1. α-Sarcin production

α-Sarcin is the best-characterized ribotoxin so far (Lacadena et al., 2007). Thus, this protein was first used to optimize the extracellular ribotoxin's production system in *L. lactis*. To this end, the α-sarcin cDNA was cloned into pT1NX plasmid fused to the usp45 lactococcal secretion signal. The initial production experiments, performed on GM9 medium, revealed the presence of an immunoreactive band in the extracellular culture medium of *L. lactis* carrying pT1αsarcin plasmid (Fig. 1). However, this band was of much higher electrophoretic mobility than wild-type α-sarcin. This suggested that *L. lactis* was able to secrete α-sarcin but also that proteolytic degradation of the recombinant protein was taking place. Considering that the final pH value of the cultures was around 4.5, in agreement with the results described by others employing similar systems (Israelsen et al., 1995; Gil et al., 2001), and that it has been reported the presence of acidic proteases within the extracellular medium of *L. lactis,* it seemed clear that the protein was being degraded. A very similar observation has been reported before for the heterologous expression of IL-10 using an almost identical system (Steidler et al., 2000). Therefore, the use of pH-buffered media was considered. Consequently, cells were grown in PM at different pH values. As can be seen in Fig. 2, production of native wild-type α-sarcin was maximal when pH values were around 8.0. Consequently, further production experiments of ribotoxins were performed in PM buffered at pH 8.0.

3.2. Toxicity of the α-sarcin producing strain on mice

Histological analysis of the three groups of mice studied (non-treated or treated with *L. lactis* carrying the cloning vector pT1NX or the expression plasmid pT1αsarcin) revealed the absence of significant differences among all of them (Fig. 3), suggesting that oral administration of ribotoxins' producing *L. lactis* strains had not deleterious effects on mice intestinal tracts.

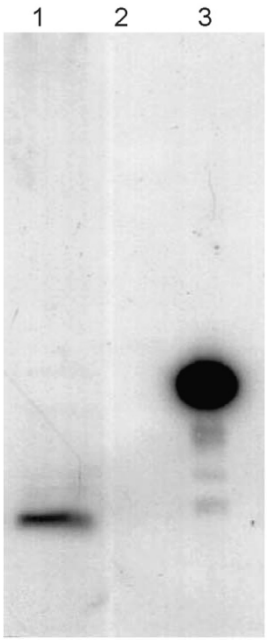

Fig. 1. Electrophoretic separation on 0.1% SDS-15% PAGE followed by Western immunoblot detection of α-sarcin in the extracellular media of overnight cultures of *L. lactis* MG1363 cells carrying the pT1αsarcin expression vector (1) or the pT1NX cloning vector (2). As a positive control, 50 ng of wild-type natural fungal α-sarcin were also loaded in lane (3).

3.3. Production of the other allergenic variants

Once the extracellular production of wild-type α-sarcin was optimized, and its lack of toxicity was assessed, four other ribotoxins' versions were also cloned and produced in *L. lactis* under identical conditions (Table 1). One of them was the wild-type major *A. fumigatus* allergen Asp f 1 (García-Ortega

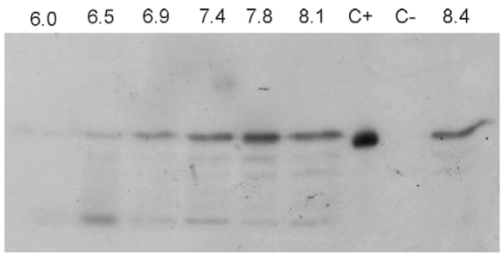

Fig. 2. Electrophoretic separation on 0.1% SDS-15% PAGE followed by Western immunoblot detection of α-sarcin in the extracellular media of buffered cultures of *L. lactis* [pT1αsarcin]. Numbers indicate the final pH values of the culture. Positive (50 ng of wild-type natural fungal α-sarcin, C+) and negative (no protein, C−) controls were also loaded.

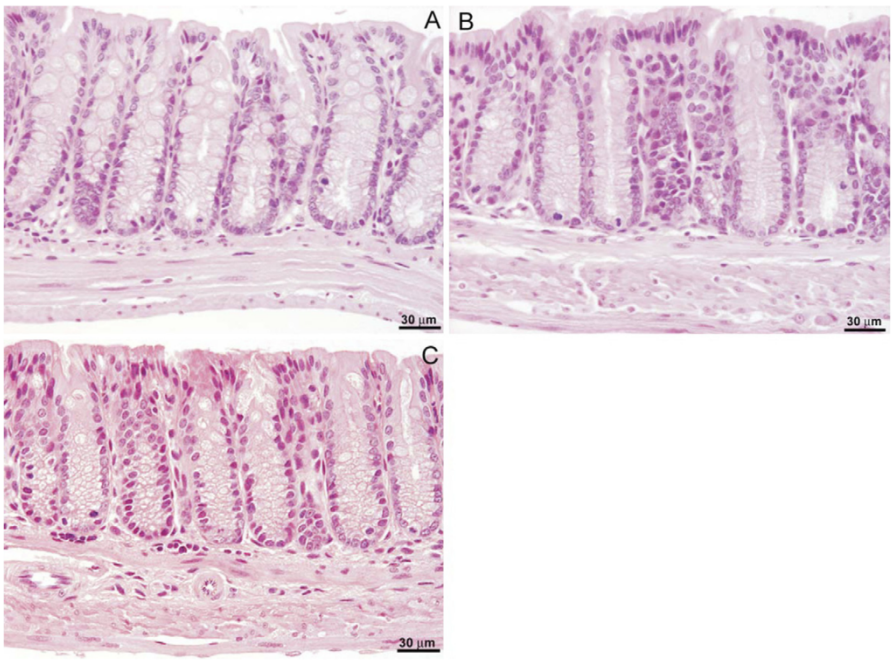

Fig. 3. H&E stains of adult mouse small intestine sections. Samples from not treated mice (A) and from mice treated with *L. lactis* strains producing (B), or not (C) α-sarcin. Note the absence of pathological signs of inflammatory response in the muscularis, submucosa, and mucosa layers in all samples. It is also observed how the epithelium lining the intestine (surface epithelium) and the glands show no discrepancies among the three different groups of animals studied. Equivalent results were obtained with sections of different areas of the large intestine (data not shown).

et al., 2005). Two other ones were the corresponding Δ(7–22) deleted variants of Asp f 1 and α-sarcin, two proteins with highly reduced IgE-reactivity and cytotoxicity (García-Ortega et al., 2002, 2005; García-Mayoral et al., 2004). The fourth one (H137Q) was a mutant version of α-sarcin where only the catalytically essential His-137 has been mutated, being substituted by Gln (Lacadena et al., 1995). This mutation renders a fully inactive and non-cytotoxic protein that however retains the wild-type native conformation (Lacadena et al., 1995, 1999). Protein production was scarcely detected in Coomassie blue stained gels (data not shown), especially for the α-sarcin variants, but it was apparent in all cases when revealed by means of Western immunoblotting and ribonucleolytic activity against poly(A). Thus, immunoreactive bands corresponding to the expected electrophoretic mobilities were observed for all the five proteins studied (Fig. 4A). Indeed, except for the catalytically inactive H137Q mutant, all the other bands corresponded to ribonucleolytically active proteins and, as proven before (García-Ortega et al., 2002, 2005), this non-specific activity was even higher for the deleted variants (Fig. 4B).

4. Discussion

There are growing evidences that regular consumption of foods containing probiotic bacteria may enhance the immune response and positively affect the indigenous microflora (Schiffrin and Blum, 2001). In addition, Gram-positive bacteria not only do not have an outer cell membrane and do not produce endotoxins but rather their peptidoglycan layer appears to exhibit natural immuno-adjuvanticity (Pouwels et al., 1996).

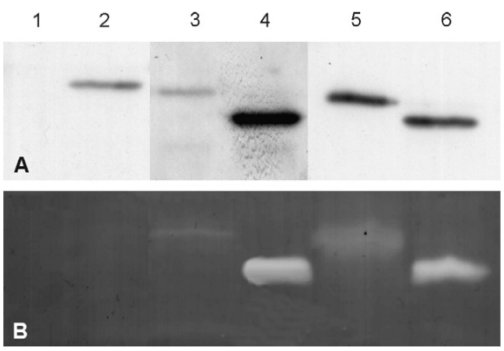

Fig. 4. (A) Electrophoretic separation on 0.1% SDS-15% PAGE followed by Western immunoblot detection and (B) zymogram against poly(A) of concentrated culture supernatants of *L. lactis* carrying pT1αsarcinH137Q (lane 2), pT1αsarcin (lane 3), pT1αsarcin7–22 (lane 4), pT1aspf (lane 5), pT1aspf 7–22 (lane 6). Lane 1 corresponds to *L. lactis* cells carrying the cloning vector pT1NX. Samples were not reduced before loading onto the gels. Immunoblot membrane corresponding to lanes 1–4 in part (A) were stained using a rabbit polyclonal serum raised against wild-type α-sarcin, whereas an anti-Asp f 1 serum was used for the area corresponding to lines 5 and 6.

This explains why oral vaccination using GRAS Gram-positive bacteria is being developed as a promising approach to the onset of different pathologies, allergies included (Pouwels et al., 1996; Robinson et al., 1997; Maassen et al., 1999; Kirjavainen et al., 1999).

L. lactis fed to animals and human volunteers passes rapidly through the gastrointestinal tract without colonization (Gruzza et al., 1994; Klijn et al., 1995). However, genetically modified versions of this microorganism are still effective in delivering antigens to the mucosal immune system and capable of inducing a local immune response (Robinson et al., 1997; Maassen et al., 1999; Adel-Patient et al., 2005; Perez et al., 2005). This seems to happen because *L. lactis* lacks the ability to multiply *in vivo* but it can readily be sampled by dendritic cells. A process that seems to be involved in the development of efficient immune responses (Xin et al., 2003), including the selective induction of IgA (Macpherson and Uhr, 2004). In addition, antigens within *Lactococci* are protected against direct contact with gastric acid and proteolytic enzymes. For example, oral administration of recombinant *L. lactis* expressing bovine β-lactoglobulin, a major cow's milk allergen, was shown to induce a specific Th1 response down-regulating a further Th2 one and thus preventing mice from sensitization (Adel-Patient et al., 2005).

Finally, only a few proteins are naturally secreted in *L. lactis*, but these bacteria can be engineered for secreting heterologous proteins to the extracellular medium, unlike most of Gram-negative bacteria in which the majority of secreted proteins are located in the periplasmic space (Nouaille et al., 2003). Altogether, all these facts make this organism optimally interesting for producing hypoallergenic proteins in order to use it as a vaccination vehicle.

Following this idea, extracellular production of five different fungal ribotoxin variants was optimized in *L. lactis* MG1363. One of these variants was the wild-type ribotoxin Asp f 1, naturally produced by the human fungal pathogen *A. fumigatus* and one of its major allergens. Three other ones are natural and artificial variants of this protein displaying a diminished reactivity against human IgE from allergic patients (García-Ortega et al., 2005). Indeed, both deleted versions [Asp f 1 and α-sarcin Δ(7–22)] show a highly reduced cytotoxicity due to the fact that they lack the ability to inactivate ribosomes specifically (García-Ortega et al., 2005). However, the four proteins secreted preserved their native conformation since all of them retained their ribonucleolytic activity against poly(A) (Fig. 4). The fifth protein produced was the α-sarcin mutant H137Q, a variant which is completely devoid of cytotoxicity and ribonucleolytic activity (Fig. 4) (Lacadena et al., 1995, 1999; Olmo et al., 2001). Additionally, neither the bacterial strain alone nor the transformed producing natural wild-type α-sarcin did induce any deleterious effect on mice intestinal tract when intragastrically administered (Fig. 3), indicating that even the highly toxic wild-type ribotoxins could be safely delivered using *L. lactis* as a vehicle for oral administration.

It is well known how gastric pH values are rather low due the presence of high HCl concentrations. However, it has been also demonstrated how a significant fraction of *L. lactis* inocula can survive and be metabolically active in all the other compartments of the intestinal tract (Klijn et al., 1995; Corthier et al., 1998; Drouault et al., 1999) where pH values are much milder, approaching neutrality in many instances (Blanquet et al., 2001). Certainly, taking into account the results shown in Fig. 2, the possibility that the observed lack of toxicity might be due just to the instability of the protein produced cannot be ruled out. Nevertheless, the overall picture is far more complex. First, it is difficult to predict the *in vivo* suitability of these approaches since many different factors seem to affect cell viability and integrity within the digestive tract compartments (Drouault et al., 1999), including the presence of the many other microorganisms composing the intestinal flora. Second, native wild-type ribotoxins are rather acid pH and proteases resistant (Martínez del Pozo et al., 1988). Third, as mentioned above, genetically engineered *L. lactis* using an identical plasmid to produce IL-10 were still therapeutically active in mice (Steidler et al., 2000) besides the fact that the *in vitro* production of this cytokine was also unstable at low pH values (Schotte et al., 2000). Finally, even if the proteins were being degraded they might be still useful as immunomodulating agents given that the resulting fragments are immunologically active (Fig. 2). Thus, in summary, it seems clear that the system described needs further evaluation in deeper detail at the *in vivo* level, especially in terms of direct detection of the activity and presence of the proteins within the mice gastrointestinal tract. However, at least within the idea of designing new immunomodulating therapeutical approaches, it seems safe to conclude that the system herein described combines the minimal features to be considered as optimally suitable for its potential application as a vehicle of oral delivery of hypoallergenic variants of Asp f 1. Finally, it has not escaped to our attention that given the antitumoral properties of ribotoxins (Olson et al., 1965; Olmo et al., 2001), this system may also constitute a starting point to approach the design of strategies to accomplish the safe delivery of these proteins as antitumoral agents against different gastrointestinal tumors.

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Discusión

Las especiales características de las ribotoxinas en términos de citotoxicidad y especificidad de acción las hacen únicas dentro de todas las ribonucleasas, a pesar de que presentan una estrecha relación estructural y filogenética con algunas de ellas, como las de la familia de la RNasa T1.

La primera característica que se pone de manifiesto en la actividad de las ribotoxinas es su capacidad para atravesar membranas celulares sin que medie ningún receptor proteico. En este sentido se ha estudiado la implicación de la horquilla β amino-terminal en este proceso, en concreto, , el papel de su carácter básico. Una vez dentro de la célula, el siguiente paso es el reconocimiento de su sustrato, el ribosoma, y en él, de un único enlace fosfodiéster en el SRL. El trabajo que se ha desarrollado ha permitido determinar la importancia de las interacciones electrostáticas que establece esta horquilla amino-terminal con el ribosoma. La última etapa en la acción de las ribotoxinas es la actividad ribonucleolítica en sí. Y a este respecto se ha estudiado la función de la Tyr 48 de la α-sarcina, un residuo conservado del centro activo.

En la célula atacada por una ribotoxina, se produce una inhibición de la biosíntesis de proteínas que provoca irremediablemente un proceso apoptótico. También se ha analizado, a nivel molecular, los efectos de esa acción ribonucleolítica que inhabilita al ribosoma para sintetizar proteínas.

Por último, se han abierto vías para futuras aplicaciones terapéuticas de estas proteínas. Por un lado, se ha considerado el posible carácter hipoalergénico de determinados mutantes de uno de los principales alérgenos de *Aspergillus fumigatus*, Asp f 1, perteneciente a la familia de las ribotoxinas, ante la posibilidad de utilizarlos en el tratamiento y prevención de procesos de hipersensibilidad a este hongo. Y, por otra parte, se han desarrollado varias cepas de *Lactococcus lactis* recombinantes con vistas a utilizarlas como vehículo para hacer llegar las ribotoxinas al tubo digestivo.

FUNCIONALIDAD DE LAS RIBOTOXINAS

Paso a través de membranas

Dentro del amplio grupo de RNasas microbianas extracelulares al que pertenecen, las ribotoxinas destacan por su mayor tamaño (alrededor de 40 aminoácidos más que las RNasas no tóxicas), su basicidad y su citotoxicidad. Este carácter citotóxico es el resultado de la conjunción de su elevada especificidad de acción ribonucleolítica y su capacidad para interaccionar con membranas (Lacadena *et al.*, 2007; Herrero-Galán *et al.*, 2008a). Desde un punto de vista estructural, las principales diferencias con respecto a las RNasas del tipo T1 radican en la longitud y ordenamiento del extremo amino-terminal y de los bucles de estructura aperiódica que conectan los elementos de estructura secundaria ordenada, aspectos que son consecuentemente considerados como determinantes de la actividad característica de las ribotoxinas. Durante años se ha estudiado la implicación de estos bucles en los procesos que confieren a las ribotoxinas sus características diferenciales, esto es, la capacidad de interacción con membranas fosfolipídicas y la especificidad frente al ribosoma. En todos los casos, los resultados han apuntado principalmente a dos zonas de la α-sarcina, el bucle 2 y la horquilla β amino-terminal. Precisamente, aunque existe un elevado grado de similitud entre las secuencias de las ribotoxinas conocidas (Rodríguez *et al.*, 1982; Sacco *et al.*, 1983; López-Otín *et al.*, 1984; Fernández-Luna *et al.*, 1985; Arruda *et al.*, 1992; Wirth *et al.*, 1997), es en estas zonas donde se observan las principales diferencias, aunque la mayoría de los cambios son conservativos.

El bucle 2 ha sido propuesto por varios autores (Yang & Moffat, 1996; Martínez del Pozo *et al.*, 1988; Kao & Davies, 1999; Pérez-Cañadillas *et al.*, 2000) como una de las regiones de la proteína implicadas en la interacción con lípidos (Figura 5b, página 15), aunque esta es una posibilidad que aún está por estudiar. Por su parte, también se han atribuido a la horquilla β amino-terminal propiedades importantes en cuanto a la interacción con lípidos. El comportamiento del mutante de deleción α-sarcina Δ(7-22) y otros mutantes puntuales (García-Ortega *et al.*, 2001,2002) en ensayos con vesículas lipídicas modelo sugieren que esta región interviene en la interacción con membranas celulares (García-Ortega *et al.*, 2001,2002).

En esta Tesis se ha estudiado la posible importancia del carácter básico de esta región amino-terminal, pues 5 de los 16 residuos que la componen son lisinas o argininas. La α-sarcina es una proteína muy cargada, con un punto isoeléctrico elevado. El 39% de su superficie está compuesta por cadenas laterales cargadas y el 26% por cadenas laterales polares (Pérez-Cañadillas *et al.*, 2000). De hecho, ya se ha demostrado en otras ribonucleasas la importancia del carácter básico en su citotoxicidad (Di Donato *et al.*, 1994; Vatzaki *et al.*, 1999; Ilinskaya *et al.*, 2002). En las ribotoxinas, la abundancia de residuos cargados positivamente cumple una doble función pues es imprescindible para el reconocimiento y unión tanto a su diana catalítica, muy cargada negativamente, el rRNA, como a las membranas celulares. Una prueba de que existe un componente

electrostático fundamental en la interacción proteína-lípido es la inhibición que se observa en la interacción entre α-sarcina y vesículas de fosfatidil glicerol al aumentar la fuerza iónica (Gasset *et al.*, 1989). Por otra parte, que la α-sarcina interaccione específicamente con vesículas de fosfolípidos cargadas negativamente a pH neutro o ligeramente ácido (Gasset *et al.*, 1989) probablemente explica su especificidad por células transformadas, con una mayor presencia de fosfolípidos ácidos en la cara externa de la membrana plasmática (Connor *et al.*, 1989; Gasset *et al.*, 1989,1990; Zachowski, 1993).

Todos estos datos llevaron a plantear que el carácter básico de la horquilla β amino-terminal podía ser fundamental en la interacción con fosfolípidos que se requiere en las primeras etapas del paso de la proteína a través de membranas. Cuando la α-sarcina se encuentra con vesículas lipídicas, interacciona electrostáticamente y mediante uniones hidrofóbicas, provocando la agregación de las vesículas seguida de la mezcla de componentes lipídicos resultante de su fusión. En las primeras fases de este proceso se forman pequeños agregados de vesículas, estabilizados por interacciones proteína-proteína y proteína-vesícula, que agregan y se fusionan dando lugar a estructuras mucho mayores (Gasset *et al.*, 1989,1990; Martínez-Ruíz *et al.*, 2001). De los cinco residuos básicos de la horquilla β amino-terminal, todos excepto la Lys 11 han resultado tener algún efecto en esa primera fase de interacción con vesículas de dimiristoilfosfatidilglicerol (DMPG). El comportamiento, en cambio, del mutante K11E es muy similar al de la proteína silvestre (Figura 9, página 84) sugiriendo que este residuo, que además no está conservado dentro de las ribotoxinas, no cumple una función muy importante en la unión de la α-sarcina a los fosfolípidos. Al estudiar la agregación de vesículas de DMPG provocada por los otros cuatro residuos estudiados, Lys 14, Lys 17, Lys 21 y Arg 22, se observan patrones muy distintos a los de la α-sarcina nativa (Figura 9, página 84), compatibles con su participación en la estabilización de esas interacciones proteína-proteína y proteína-vesícula que provocan la agregación inicial de vesículas. Estos resultados sugieren, además, que estas interacciones son, al menos en parte, de carácter electrostático. Las diferencias más notables con la proteína silvestre las muestran los mutantes de Lys 14 y Lys 21 (Figuras 9 y 10, páginas 84 y 85), que se orientan en una misma dirección, ortogonal a Lys 17 y Arg 22 y opuesta a Lys 11 (Figura 1, página 79). De hecho, el cambio en esas posiciones de una carga positiva por una negativa provoca efectos cualitativamente distintos a los causados por las mutaciones en los residuos 17 y 22, pues no sólo retardan la primera fase (que equivale a la formación de pequeños agregados de vesículas fosfolipídicas) sino también la segunda (correspondiente a la unión y/o fusión de esos pequeños agregados) (Figura 9, página 84). Las dos cargas positivas orientadas en esa dirección parecen, por tanto, esenciales en el establecimiento de uniones electrostáticas requeridas para la interacción de la α-sarcina con las membranas fosfolipídicas.

Como se ha comentado, parece que la interacción que la α-sarcina establece con la membrana celular que va a atravesar se forma principalmente a través de la horquilla β amino-terminal y del bucle 2. Los abundantes residuos básicos de estas regiones están orientados de forma que permiten proponer un modelo de unión de la proteína a la membrana como el que muestra la figura 8, en el que las cadenas laterales de estos aminoácidos básicos establecerían interacciones electrostáticas con las cabezas polares de fosfolípidos ácidos.

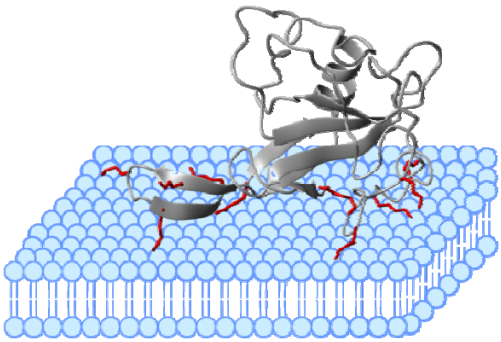

Figura 8. Modelo de interacción entre la α-sarcina y una bicapa de fosfolípidos ácidos. Se señalan en rojo algunos de los residuos básicos (lisinas y argininas) de la horquilla β amino-terminal y del bucle 2 de la proteína, posiblemente implicados en uniones electrostáticas con las cabezas polares de los fosfolípidos. El diagrama de la α-sarcina fue generado con el programa MOLMOL (Koradi *et al.*, 1996).

Reconocimiento del ribosoma

Además de la capacidad para interaccionar con fosfolípidos y atravesar membranas celulares, la exquisita especificidad ribonucleolítica de las ribotoxinas es la otra característica que las distingue del resto de las RNasas extracelulares microbianas. Esta especificidad se debe a un reconocimiento sutil y muy específico que les permite degradar un único enlace fosfodiéster de todos los presentes en el ribosoma. Obviamente las ribotoxinas deben reconocer el SRL, donde se encuentra este enlace objeto de su acción enzimática, pero también debe de haber otros elementos de reconocimiento, distantes espacialmente del SRL, que interaccionen con ellas. De nuevo, el extremo amino-terminal y los largos bucles de estructura aperiódica, los elementos estructurales más característicos de las ribotoxinas, parecen ser los principales responsables en el reconocimiento del ribosoma.

Dentro de las interacciones que la α-sarcina establece directamente con el SRL, se cree que la más determinante es la que forma una región rica en Lys (Lys 111, Lys 112 y Lys 114) del bucle 3 con el enlace fosfodiéster cargado negativamente en los alrededores de la G prominente, el elemento más identificativo del SRL (Figura 9) (Yang &Moffat, 1996; Pérez-Cañadillas *et al.*, 2000; Yang *et al.*, 2001). Por otra parte, la otra interacción propuesta, entre los residuos 52-55 del bucle 2 y el tetrabucle GAGA conservado que contiene el enlace que hidroliza la toxina (Pérez-Cañadillas *et al.*, 2000), parece confirmada por cristalografía de rayos X (Yang *et al.*, 2001) (Figuras 9 y 5a, página 15). Pero el papel del bucle 2 en el reconocimiento del ribosoma no termina aquí. Sus características, en cuanto a movilidad, basicidad y exposición al disolvente, le confieren importantes implicaciones funcionales (Pérez-Cañadillas *et al.*, 2000). En este sentido, mediante la modelización del reconocimiento de las ribotoxinas por el ribosoma, se ha propuesto que debe de existir una interacción entre una pequeña secuencia extendida de este bucle y la proteína ribosomal L6 (Figura 5c, página 15) (García-Mayoral *et al.*, 2005b).

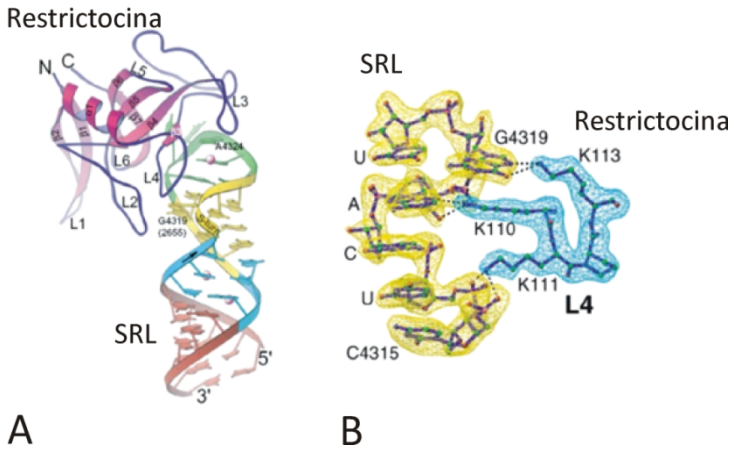

Figura 9. (A) Diagrama de cintas del complejo restrictocina-SRL. En el SRL se muestra en verde el tetrabucle GAGA y en amarillo la G prominente. Los bucles L1, L2, L3, L4 y L5 de la restrictocina se corresponden con la horquilla β amino-terminal, y los bucles 1, 2, 3 y 4 de la α-sarcina, respectivamente. (B) Contactos entre Lys 110, Lys 111 y Lys 113 de la restrictocina (correspondientes a Lys 111, Lys 112 y Lys 114 del bucle 3 de la α-sarcina) con el giro del SRL que incluye a la G prominente. Figura tomada de Yang *et al.*, 2001.

La horquilla β amino-terminal es otro elemento esencial para el correcto reconocimiento del ribosoma, al igual que para la interacción con lípidos y el paso a través de membranas. Como el bucle 2, este elemento estructural presenta características de basicidad y exposición al disolvente que parecen ser claves en su funcionalidad. El mutante de deleción α-sarcina Δ(7-22) (García-Ortega *et al.*, 2001,2002) proporcionó información muy valiosa en este aspecto. Este mutante, con la misma conformación que la proteína nativa y con capacidad para hidrolizar específicamente oligonucleótidos que mimetizan el SRL, no reconoce al ribosoma como sustrato específico (García-Ortega *et al.*, 2002). Estudios recientes apuntan a que, una vez internada en la célula, el carácter básico de la α-sarcina hace que rápidamente encuentre al ribosoma, de naturaleza ácida, y entonces difunda por su superficie hasta colocarse en la posición adecuada para hidrolizar el SRL, igualando la eficiencia catalítica de las enzimas más rápidas conocidas (Korennykh *et al.*, 2006; Plantinga *et al.*, 2008). Para encontrar esta posición debe establecer interacciones con distintas regiones del ribosoma, no sólo con el SRL, pues la velocidad con la que la α-sarcina hidroliza el ribosoma completo es unas 1000 veces mayor que la que muestra con análogos del SRL (Korennykh *et al.*, 2006; Endo *et al.*, 1988). Estas interacciones pueden determinar que, a pesar de que el SRL esté muy conservado tanto en secuencia como en estructura (García-Mayoral *et al.*, 2005; Korennykh *et al.*, 2006), la eficacia en la actividad de la ribotoxina frente a ribosomas de distintas fuentes biológicas sea diferente (Schindler y Davies, 1977; Miller y Bodley, 1991). Por ejemplo, es bien conocido que los ribosomas de *E. coli*, y los de los procariotas en general, son menos susceptibles a la acción de la α-sarcina que ribosomas de organismos eucariotas (Schindler y Davies, 1977; Endo y Wool, 1982; Hausner *et al.*, 1987). En el trabajo aquí presentado se ha tratado este aspecto ya que se ha

demostrado que, además de esta menor eficacia, la α-sarcina muestra una menor especificidad por estos ribosomas, ya que no sólo hidroliza el enlace entre la G2661 y la A2662, sino también el contiguo en el lado 3' (Figura 1, página 108). Esta menor especificidad y eficacia pueden deberse a un peor reconocimiento del ribosoma por parte de la α-sarcina, en lugares distintos, pero próximos, al SRL, aunque tampoco se pueda descartar que las condiciones de reacción empleadas se alejen tanto de las fisiológicas, sobre todo en cuanto a concentración de iones divalentes, que la conformación del SRL se esté viendo afectada.

Volviendo a la horquilla β amino-terminal, cualquier comparación entre las distintas ribonucleasas microbianas extracelulares (Figura 10) pone de manifiesto que es el elemento estructural más característico de las ribotoxinas. Esta protuberancia que, como se ha comentado, puede ser eliminada sin distorsionar ni la estructura global de la enzima, ni su actividad catalítica (García-Mayoral *et al.*, 2004; García-Ortega *et al.*, 2002), debe de cumplir una función importante. En consonancia con esta idea, su deleción le impide reconocer específicamente el ribosoma. Dicha variante, sin embargo, sigue siendo capaz de hidrolizar específicamente análogos del SRL, sugiriendo que al eliminar la horquilla no se eliminan los elementos de reconocimiento de esta región del rRNA, sino que son otras interacciones, con otras zonas del ribosoma, las que dejan de tener lugar (García-Ortega *et al.*, 2002).

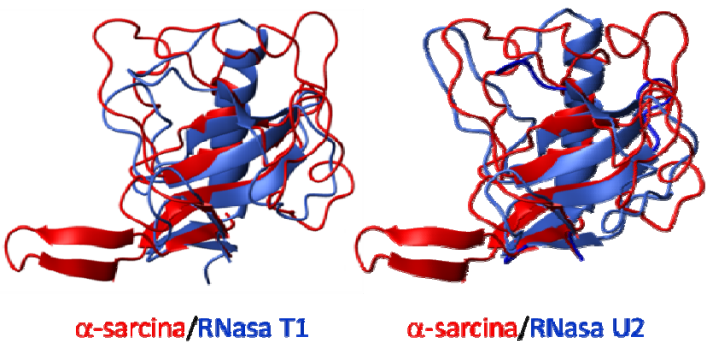

Figura 10. Superposición de las estructuras tridimensionales de la α-sarcina (en rojo) con las de las RNasas microbianas no tóxicas más representativas (en azul), la RNasa T1 y la U2. Diagramas generados con el programa MOLMOL (Koradi *et al.*, 1996).

Mediante la modelización de la interacción que tendría lugar entre la α-sarcina y el ribosoma de *Haloarcula marismortui*, se predijo que la horquilla participaría en interacciones específicas mediadas por la proteína ribosomal L14 (Figura 5c, página 15)(García-Mayoral *et al.*, 2005b) y que las interacciones de carácter electrostático serían importantes en este reconocimiento (García-Mayoral et al., 2005). Al menos este segundo aspecto parece ya probado tras la obtención de los resultados que se presentan en esta Memoria. Al cambiar cada una de las cargas positivas presentes en la horquilla β amino-terminal (posiciones 11, 14, 17, 21 y 22), y sustituirlos por cargas negativas, no se altera la actividad frente a análogos del SRL o poli A (Figuras 7 y 8, página 83) pero sí

se ve sensiblemente afectada su acción frente a ribosomas completos (Figura 6, página 82). La Lys 11 y la 14 son los dos residuos que precisamente se habían predicho como presuntamente involucrados en la interacción con la proteína ribosomal L14 (García-Mayoral *et al.*, 2005b). El hecho de que la lisina 11 sea el único residuo no conservado en las ribotoxinas conocidas, y que su sustitución por Leu (el residuo presente en la restrictocina) no altere su capacidad para reconocer específicamente los ribosomas (García-Ortega *et al.*, 2001) permiten especular acerca de su diferente papel en el reconocimiento de ribosomas de orígenes distintos.

Más sorprendente aún resulta la observación de que de los 5 mutantes caracterizados, el correspondiente a la Lys 14 mostró además un cambio muy significativo a la hora de reconocer sustratos menos específicos. Así, este mutante K14E se mostró capaz de degradar poliC en condiciones en las que ni la proteína silvestre, ni ninguno de los otros cuatro mutantes puntuales ensayados son capaces de hacerlo. Desde que se resolvió la estructura tridimensional de la α-sarcina (Pérez-Cañadillas *et al.*, 2000) se presume que algunas de las interacciones que se establecen entre residuos de la horquilla y residuos de la lámina β central serían claves para mantener la especial configuración del centro activo de la α-sarcina. Sin ir más lejos, parece probado que precisamente la interacción electrostática que se establecería entre las cadenas laterales de la Lys 11 y el Glu 140 sería una de las más importantes en este sentido (Pérez-Cañadillas *et al.*, 2000). Este mismo Glu 140 forma, a su vez, un puente de hidrógeno con el Asp 9, mientras que el residuo catalítico His 137 interacciona con la Asn 8. Por último, ya hace años que se demostró que la sustitución de esta Asn por Ala rendía un mutante de restrictocina mucho menos específico (Kao y Davies, 2001). Es decir, a pesar de ser un pequeño dominio estructural que se puede eliminar sin alterar drásticamente las propiedades de la enzima, la horquilla β amino-terminal parece estar involucrada en toda una red de interacciones que mantienen su conformación en la configuración óptima para su acción letal. El conjunto de resultados presentado, y muy especialmente el concerniente al mutante K14E de la α-sarcina, confirmarían esta hipótesis. La reversión de carga en esta posición tendría la suficiente trascendencia sobre la mencionada red de interacciones como para alterar la distribución espacial del centro activo, o su accesibilidad, provocando un cambio muy significativo en su capacidad para degradar homopolinucleótidos.

Mecanismo catalítico. El centro activo de las ribotoxinas

El centro activo de las ribotoxinas constituye otra de sus características diferenciales. Por un lado, mantiene una configuración espacial muy parecida a la de todas las demás ribonucleasas microbianas extracelulares (Figura 4, página 12) pero, por otro, reúne unas propiedades muy especiales en términos de constante dieléctrica y accesibilidad al disolvente.

Los residuos clave del centro activo de las ribotoxinas se identificaron por comparación con la RNasa T1. A pesar de sus diferencias, entre las que destacan la baja especificidad y la falta de

citotoxicidad de ésta, son proteínas extremadamente parecidas, en las que los residuos del centro activo se superponen perfectamente (Figura 11). El centro activo de la RNasa T1, la más estudiada de todas las RNasas extracelulares microbianas, está perfectamente caracterizado (Steyaert, 1997). En la reacción global (la producción de un nucleótido cíclico seguida de su hidrólisis) (Figura 7, página 21) intervienen el Glu 58 y la His 92. Durante la primera reacción, la formación del producto cíclico 2',3', el Glu 58 actúa como base general y la His 92 como ácido general. La hidrólisis de este derivado cíclico la llevan a cabo los mismos grupos, pero con los papeles intercambiados. Además, hay otra histidina, en posición 40, con un papel crítico, que asiste a la catálisis ejercida por el Glu 58, probablemente orientando el ataque nucleofílico sobre el fósforo (Steyaert, 1997). En la α-sarcina, el Glu 96, la His 137 y la His 50 son los equivalentes estructurales y funcionales a Glu 58, His 92 e His 40 de la RNasa T1 (Lacadena *et al.*, 1999; Martínez-Ruiz *et al.*, 2001). El centro activo de la RNasa T1 contiene tres aminoácidos más, Tyr 38, Arg 77 y Phe 100, que aunque no están implicados en los pasos de transferencia de protones contribuyen a la catálisis formando un microambiente dieléctrico complementario en forma y carga al estado de transición y contribuyendo a su óptima solvatación/desolvatación (Loverix y Steyaert, 2001). La cadena lateral de la Phe 100 es un elemento catalítico apolar, que controla el entorno dieléctrico estabilizando las separaciones de carga que aparecen en el estado de transición (Doumen *et al.*, 1996). Se cree que la Arg 77 facilita el ataque nucleofílico, aunque esto nunca se ha podido probar pues todos los intentos de producir un mutante

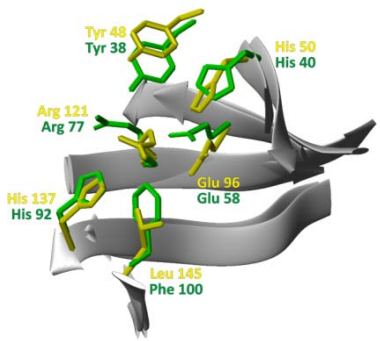

Figura 11. Superposición de la estructura de la lámina β central de la RNasa T1 y la α-sarcina. También se superponen los residuos que componen el centro activo de ambas. En verde aparecen los residuos correspondientes a la RNasa T1 y en amarillo, a la α-sarcina. El diagrama se generó con el programa MOLMOL (Koradi *et al.*, 1996).

en el que se sustituyese esa Arg han resultado fallidos (Steyaert, 1997). Por último, la Tyr 38 de la RNasa T1 estabiliza al estado de transición al formar un enlace de hidrógeno con él.

Los equivalentes a estos residuos de la RNasa T1 (Phe 100, Arg 77 y Tyr 38) en la α-sarcina son la Leu 145, la Arg 121 y la Tyr 48 (Figura 11). En esta Tesis se ha estudiado el papel de la Tyr 48, el único de estos residuos que quedaba sin analizar. Para ello, se ha sustituido este residuo por una fenilalanina. La caracterización estructural del mutante Y48F mostró que conserva la conformación nativa. Los ensayos funcionales que siguieron a esta caracterización estructural se dividieron en dos bloques. Por un lado, se analizó la capacidad de interacción con fosfolípidos, midiendo la agregación de vesículas de DMPG provocada por el mutante. Así se comprobó que la sustitución de la Tyr 48 por Phe no modifica la capacidad de la α-sarcina para interaccionar con fosfolípidos y así penetrar en sus células diana (Figura 7, página 74). Esta tirosina se encuentra en una zona, la lámina β central de la proteína, muy importante en esa interacción, pues constituye el núcleo con el que interacciona la

parte hidrofóbica de la bicapa lipídica. Muy próxima, por ejemplo, se encuentra la Arg 121 (Figura 1, página 71), también constituyente del centro activo, cuya carga positiva es esencial en la interacción con fosfolípidos (Masip *et al.*, 2001). Se podría interpretar que la Tyr 48 no forma parte de ese grupo de residuos en la lámina β de la α-sarcina que son decisivos para su paso a través de membranas, así como tampoco lo es, por ejemplo, el Trp 51, (De Antonio *et al.*, 2000). Pero debemos considerar que Trp 51 y Tyr 48 tienen orientaciones prácticamente opuestas dentro de esta lámina β (Figura 12) y que en el mutante Y48F la tirosina se ha sustituido por una fenilalanina, un residuo aún más hidrofóbico. Así, continúa siendo probable que la Tyr 48 sí intervenga en la interacción con las bicapas lipídicas.

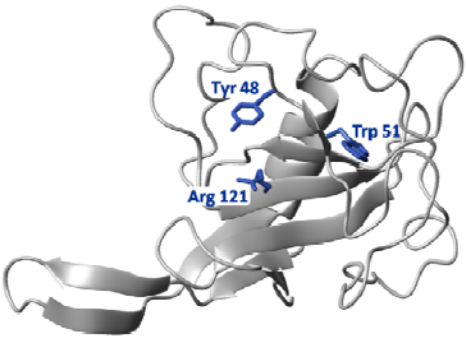

Figura 12. Diagrama de la α-sarcina en la que se destaca la orientación geométrica de los residuos Tyr 48, Trp 51 y Arg 121. Diagrama generado con el programa MOLMOL (Koradi *et al.*, 1996).

Cuando se estudió la funcionalidad del mutante en lo que a actividad catalítica se refiere, los resultados fueron muy diferentes. El cambio de Tyr por Phe, que únicamente supone la eliminación de un grupo hidroxilo, es suficiente para abolir la capacidad de la proteína de reconocer sustratos poliméricos, incluyendo al sustrato natural, el ribosoma (Figura 6, página 73). En cambio, la capacidad para hidrolizar enlaces fosfodiéster en general se ve poco afectada, pues la eficiencia catalítica frente a ApA se mantiene muy similar a la de la proteína silvestre (Tabla 2, página 73). En resumen, la Tyr 48 ejerce un papel clave en la especificidad de la α-sarcina. Ya se menciona en la Introducción que las ribonucleasas que actúan frente a sustratos de RNA poliméricos deben distorsionar la orientación de las bases (Yang *et al.*, 2001), y que esta función en las ribotoxinas parece llevarla a cabo el triplete Tyr 48, Arg 121 y Leu 145. El hecho de que el mutante Y48F hidrolice perfectamente ApA (Tabla 2, página 73), e incluso hidrolice de forma inespecífica análogos sintéticos del SRL (datos no mostrados) siendo, en cambio, incapaz de reconocer específicamente al ribosoma (Figura 6, página 73), indica que, efectivamente, la Tyr 48 cumple un papel esencial facilitando a la α-sarcina romper el SRL en el sitio adecuado.

Los resultados obtenidos con el mutante de la Tyr 48 son muy similares a los de Arg 121 y Leu 145. También la sustitución de estos residuos provoca una disminución notable en la especificidad de la ribotoxina. En estos casos, además, la constante catalítica de la enzima disminuye alrededor de un orden de magnitud. Resulta muy interesante que tres residuos que en la RNasa T1 mejoran la catálisis de reacciones de ruptura inespecífica de RNA, en la α-sarcina cumplen un papel

mucho más completo. No sólo aumentan la capacidad catalítica (en todos los casos, excepto en el de la Tyr 48 los mutantes exhiben constantes catalíticas sensiblemente menores a la de la proteína silvestre) sino que, además, proporcionan especificidad a la α-sarcina, permitiendo el reconocimiento de un único enlace fosfodiéster en todo el ribosoma.

El centro activo de la RNasa U2

Dentro de las ribonucleasas extracelulares no tóxicas, la RNasa U2, producida por el hongo *Ustilago sphaerogena*, es la más cercana a las ribotoxinas. Posee alrededor de 10 aminoácidos más que el resto de proteínas de su familia (114 frente a 101-106) y su identidad de secuencia con las ribotoxinas es del 34%, lo que la sitúa a la menor distancia filogenética respecto a ellas. Los aminoácidos adicionales que la RNasa U2 presenta con respecto a la T1 se localizan principalmente en los bucles externos, siendo algo más largos los bucles 2 (44-59) y 3 (65-83) y el extremo amino-terminal, características que, de nuevo, la sitúan más cerca de las ribotoxinas. Por todo esto, lleva años empleándose en nuestro grupo como el mejor modelo comparativo entre las ribotoxinas y las RNasas de la familia T1. En cuanto a su mecanismo catalítico, está demostrado que también se trata de una RNasa ciclante, aunque con una especificidad bastante inusual dentro del grupo de ribonucleasas microbianas extracelulares al que pertenece, pues prefiere purinas en el extremo 3' del enlace que hidroliza, y no sólo guaninas, como el resto de las RNasas tipo T1 (Uchida *et al*., 1970). Otra particularidad es su intervalo funcional de pH, entre 3.5 y 5.5 (Arima *et al*., 1968b). Pero a pesar de estas diferencias, la RNasa U2 mantiene el mismo plegamiento general que el resto de miembros de su familia, superponiéndose de manera casi perfecta con la RNasa T1 (Figura 1, página 89). Esta superposición, junto con estudios de modificación química, permitió hace tiempo proponer que los residuos catalíticos del centro activo serían el Glu 62, la His 101 y la His 41 (Sato y Uchida, 1975b,c; Egami *et al.*, 1980; Noguchi *et al.*, 1995).

Con el objetivo de verificar que, efectivamente, la His 101 de la RNasa U2 es el residuo que actúa como ácido general durante la formación del intermedio cíclico en la ruptura del RNA, se produjo y purificó a homogeneidad un mutante puntual en el que este residuo se sustituyó por glutamina (Figuras 2 y 3, página 94). Pero la información proporcionada por este mutante fue muy distinta a la esperada. El dato más interesante es el sorprendente papel esencial que posee este residuo en el plegamiento de la proteína, papel que no comparten los residuos equivalentes de la RNasa T1 ni de la α-sarcina. En ambas proteínas, la histidina catalítica correspondiente (His 92 e His 137 respectivamente) se ha mutado obteniéndose proteínas con conformación nativa (Lacadena *et al.*, 1999; Steyaert y Wyns, 1993). Sin embargo, la sustitución de la His 101 en la RNasa U2 provoca un plegamiento incorrecto de la proteína, lo que, por otra parte, impide hacer ninguna afirmación en cuanto a su mera función catalítica. La conformación del mutante se analizó mediante dicroísmo circular y emisión de fluorescencia, viéndose reflejada por ambas técnicas la adopción de una estructura no nativa. El espectro de dicroísmo circular de U2 H101Q tiene una forma completamente distinta al correspondiente a la proteína nativa y perfectamente compatible con un alto contenido en estructura secundaria aperiódica. En cuanto a la fluorescencia, el espectro de emisión de la

proteína silvestre excitada a 275 nm está dominado por la emisión de las tirosinas, quedando la emisión de un triptófano presente en la proteína fuertemente apagada. En el mutante U2 H101Q la emisión de este triptófano se hace evidente. Y no sólo cambia la emisión de este residuo sino que también aumenta el rendimiento cuántico de las tirosinas (Figura 7, página 97), sugiriendo que en la estructura nativa éstas también se encuentran apagadas. Este hecho recuerda a lo observado con una fracción que aparece cuando se purifica la proteína silvestre producida en *Pichia pastoris*. En este caso, sólo es una fracción la que no se pliega correctamente, pudiendo obtenerse en forma nativa alrededor de 1 mg de RNasa U2 silvestre por litro de cultivo (tabla 2, página 92). Sin embargo, la sustitución de la His 101 impide completamente la adopción de la conformación nativa, y la totalidad de la proteína obtenida aparece desnaturalizada. Este resultado demuestra, por tanto, el carácter esencial de la His 101 en el plegamiento de la RNasa U2.

La adopción de una conformación no nativa tiene como consecuencia, además, que varias características de la proteína, como el procesamiento del extremo amino-terminal o modificaciones postraduccionales, se vean afectadas. En primer lugar, el mutante U2 H101Q constituye un ejemplo del carácter impredecible de las glicosilaciones introducidas por *Pichia pastoris*. En toda la secuencia primaria de la RNasa U2 no aparece ninguna secuencia consenso de glicosilación en Asn, pero hace unos años se observó que *Pichia pastoris* también es capaz de O-glicosilar las proteínas que expresa (Cereghino & Cregg, 2000; Tsujikawa *et al*, 1996; Juge *et al*, 1996; Heimo *et al*, 1997). No parece que exista ninguna secuencia consenso de aminoácidos para las O-glicosilaciones, en las que los eucariotas inferiores unen residuos de manosa al grupo hidroxilo de residuos de serina y treonina. Además, está descrito que proteínas, como el factor de elongación I similar a insulina (IGF-I, *insulin-like growth factor I*), que no están glicosiladas en humanos, unen residuos de manosa cuando son expresadas de forma heteróloga en *Pichia pastoris* (Brierley, 1998). Ni siquiera tienen por qué ser los mismos residuos de Ser y/o Thr glicosilados los que se glicosilan en *Pichia pastoris* y en la fuente natural de la proteína. Con todos estos datos, no es extraño que cuando la conformación de la RNasa U2 varía al sustituir la His 101 por glutamina la levadura encuentre algún residuo susceptible de unir azúcares. Precisamente, la His 101 de la RNasa U2 está flanqueada por dos residuos de Thr (en posiciones 100 y 102) que podrían pasar de un entorno protegido, cuando la proteína está en su conformación nativa, a quedar expuestos a glicosilaciones en el mutante. Esta hipótesis cobra aún más sentido si se considera que estos tres residuos forman parte de una hebra β en la que His 101 estaría hacia el disolvente, expuesta, pues forma parte del centro activo, mientras que las Thr se orientarían en la dirección opuesta, mucho menos accesibles (Figura 13). Otra posibilidad alternativa, que también debe tenerse en cuenta, sería que la sustitución de la His 101 por Gln cambiase sólo ligeramente el entorno de las treoninas pero lo suficiente como para permitir su glicosilación y que esta glicosilación fuese la verdadera causa del mal plegamiento de la proteína.

Otro efecto inesperado de la sustitución de la His 101 por glutamina fue la observación de que el procesamiento del extremo amino-terminal se veía afectado. Cuando se expresa la RNasa U2 silvestre en *Pichia pastoris*, la estructura terciaria de la proteína y la presencia de un puente disulfuro en el que interviene una cisteína en posición 1 impiden que los mecanismos proteolíticos procesen adecuadamente el péptido señal del factor-α de *Saccharomyces cerevisiae*, que es el que dirige a la

RNasa U2 al medio extracelular. Concretamente, se ve afectada la última etapa de ese procesamiento, en la que interviene la proteasa Ste13 eliminando repeticiones de Glu-Ala (Brake *et al.*, 1984). Así, la RNasa U2 expresada en *Pichia pastoris* presenta 6 aminoácidos adicionales en el extremo amino-terminal: Glu-Ala-Glu-Ala-Glu-Leu (Martínez-Ruiz *et al.*, 2000; García-Ortega *et al.*, 2005b). Al secuenciar el extremo amino terminal del mutante H101Q se observó que el péptido señal tampoco se había eliminado por completo, pero en este caso no se encontró una única secuencia. Además de los 6 aminoácidos extra que se encuentran en la proteína silvestre, aparecen dos secuencias más, con sólo 5 ó 2 aminoácidos extra (Figura 4, página 95). Probablemente el mutante, más desplegado que la proteína silvestre, deja más accesibles los sitios de corte de la proteasa, permitiendo una mayor variedad de cortes y un procesamiento postraduccional mucho menos homogéneo.

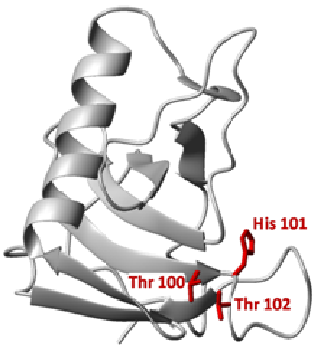

Figura 13. Diagrama de la estructura tridimensional de la RNasa U2 en el que se destaca la disposición geométrica de los residuos Thr 100, His 101 y Thr 102. Diagramas generados con el programa MOLMOL (Koradi et al., 1996).

El patrón de puentes disulfuro de las ribotoxinas

Un punto importante en el estudio de las relaciones estructura-función de las ribotoxinas es el patrón de puentes disulfuro, por su implicación en la estructura y estabilidad de las proteínas. De hecho, en muchos casos, las relaciones filogenéticas de las proteínas han sido determinadas en primera instancia a partir de dicho patrón (Irie, 1997). Dentro de la familia de las ribotoxinas aparecen dos puentes disulfuro perfectamente conservados (Lacadena *et al.*, 2007). El primero de ellos, el que une los extremos amino y carboxilo terminales, aparece también en todas las RNasas de la familia T1, mientras que el segundo, más interno, reproduce el que adicionalmente presentan los miembros de esta familia producidos por hongos basidiomicetos (RNasas U1, U2 y Po1) (Wool, 1997; Martínez-Ruíz *et al.*, 1999b; Kao *et al.*, 2001). Fijándonos en esta disposición, de nuevo la RNasa U2 se encuentra en un punto intermedio entre las RNasas no tóxicas de la familia de la T1 y las ribotoxinas. Además de estos dos enlaces mencionados (que se establecen entre los residuos 9 y 113, y 55 y 96, respectivamente) presenta un tercero con su equivalente en las RNasas no tóxicas, pero no en las ribotoxinas (entre las cisteínas 1 y 54) (Figura 14) (Sato y Uchida, 1975d).

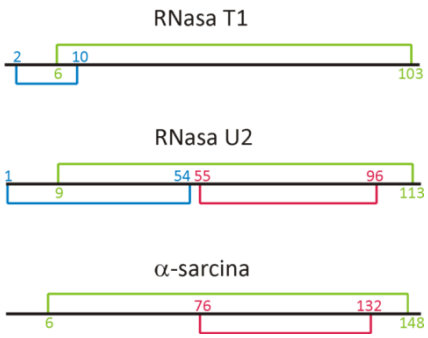

Figura 14. Patrón de puentes disulfuro en las RNasas T1, U2 y α-sarcina. El mismo color indica equivalencia del enlace en las distintas secuencias.

Este último puente disulfuro merece un estudio especial por varios motivos. En primer lugar, por ser el único de los conservados en la familia de la RNasa T1 que no aparece en las ribotoxinas. También es interesante porque la existencia de una cisteína en posición 1 de la secuencia primaria de la RNasa U2 podría impedir que el extremo amino terminal se procese correctamente cuando se expresa de forma heteróloga en *Pichia pastoris* (Martínez-Ruíz *et al.*, 2000; García-Ortega *et al.*, 2005b). Por último, como ya se ha mencionado, al expresar la RNasa U2 de forma recombinante en *Pichia pastoris* aparece una fracción de proteína mal plegada, y existía un especial interés por comprobar si el intercambio de las Cys 54 y 55 en los puentes disulfuro en los que están implicadas era la causa de ese mal plegamiento. Dada la orientación y proximidad de esos dos puentes disulfuro (Figura 15) no puede descartarse que en esa fracción de la proteína se formen los enlaces entre las cisteínas 1-55 y 54-96, en vez de los nativos 1-54 y 55-96 (Figura 15).

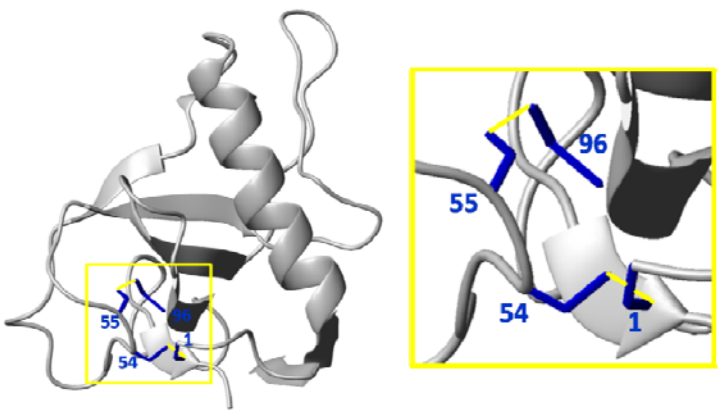

Figura 15. Diagrama de la estructura de la RNasa U2 y detalle de dos de sus puentes disulfuro, entre los pares de cisteínas 1-54, y 55-96. Diagramas generados con el programa MOLMOL (Koradi *et al.*, 1996).

Para intentar dar respuesta a estas preguntas se produjo un doble mutante de las Cys 1 y 54 de la RNasa U2, en el que faltaba ese puente disulfuro. Su expresión en *Pichia pastoris*, y su purificación desveló que, estructuralmente, este mutante doble presenta características equivalentes a las de la proteína silvestre, excepto en dos detalles poco sorprendentes. Por un lado,

su estabilidad térmica, con un puente disulfuro menos en su estructura, resultó ser mucho menor que la de la proteína silvestre (Tabla 2, página 92). Por otra parte, como habíamos esperado, la sustitución de la cisteína en posición 1 por una serina permitió que el procesamiento del péptido señal en *Pichia pastoris* se produjera correctamente. Las proteasas que eliminan los últimos aminoácidos de ese péptido no encuentran impedimentos cuando no existe ese puente disulfuro, obteniéndose una secuencia primaria de la misma longitud que la de la proteína silvestre. En cuanto a la capacidad enzimática del mutante, no se observan grandes diferencias con lo medido para la proteína nativa, hecho que concuerda con la equivalencia estructural observada. Lo más llamativo de este mutante se observó en el momento de su purificación, en la que, a diferencia de la proteína silvestre, no se detectó ninguna fracción de proteína con un plegamiento no nativo y con características espectroscópicas distintas a las nativas, lo que parece confirmar la hipótesis de que el patrón de puentes disulfuro tiene algo que ver con la aparición de esta fracción de proteína desnaturalizada.

IMPLICACIÓN BIOLÓGICA DE LA ACCIÓN DE LAS RIBOTOXINAS EN LA FUNCIONALIDAD DEL RIBOSOMA

Desde que se descubrió a finales de los años 70 la exquisita actividad ribonucleolítica de las ribotoxinas (Schindler y Davies, 1977; Endo y Wool, 1982), parte de los estudios sobre ellas se ha centrado en la implicación biológica de su actividad, esto es, en cómo afecta la actividad de la toxina al ribosoma y a la célula en general. Además del valor del conocimiento en sí mismo, no cabe duda de que profundizar en este aspecto puede ser de gran utilidad en un futuro uso de estas proteínas como agentes terapéuticos.

La α-sarcina se interna en la célula mediante un mecanismo de endocitosis, en un transporte independiente de clatrina en el que pasa por endosomas ácidos y el aparato de Golgi antes de llegar al citosol (Olmo *et al.*, 2001), siendo el cruce de la membrana plasmática el paso limitante en la actividad citotóxica de la proteína (Turnay *et al.*, 1993). Ya en el citosol, actúa sobre los ribosomas, provocando la inhibición de la biosíntesis de proteínas, que lleva a la célula a morir por apoptosis (Olmo *et al.*, 2001). Profundizando en las consecuencias del corte producido por la α-sarcina, se ha estudiado su efecto en la funcionalidad del ribosoma.

Sin duda, la zona donde se encuentra el enlace hidrolizado por la α-sarcina no es casual. La ribotoxina ataca un punto débil del ribosoma, el SRL, con un papel funcional tan importante que mutaciones en su secuencia provocan en efectos letales en las células (Macbeth y Wool, 1999; Chan *et al.*, 2000). Ese carácter esencial del SRL se explica por formar parte del sitio de unión de los factores de elongación EF-G y EF-Tu (Moazed *et al.*, 1988; Valle *et al.*, 2003a,2003b). Por eso este estudio se centró en la fase de elongación dentro de la biosíntesis de proteínas, que previsiblemente sería la fase afectada por el corte de la α-sarcina.

En general, todos los trabajos en los que se ha analizado el efecto del corte de las ribotoxinas sobre el ribosoma apuntan como principales consecuencias a una unión deficiente de los factores de elongación (Fernández-Puentes y Vázquez, 1977; Hausner *et al.*, 1987; Brigotti *et al.*, 1989; Miller y Bodley, 1991; Nierhaus *et al.*, 1992). Y los más recientes, a que la función de EF-Tu no se ve modificada sino únicamente la de EF-G (Blanchard *et al.*, 2004), sugiriendo que el SRL no interacciona de la misma forma con los dos factores de elongación. De hecho, aunque los ribosomas tratados con α-sarcina conservan la capacidad de unir el complejo ternario (EF-Tu•GTP•aa-tRNA) y catalizar la formación del enlace peptídico (Figura 3, página 110), son incapaces de sufrir la translocación dependiente de EF-G (Figura 4, página 111), que dejaría al ribosoma listo para la incorporación de un nuevo complejo ternario al sitio A. Dentro de todas las etapas que conforman esta translocación, se ha determinado que la que se ve afectada por la ruptura del SRL es la inicial, la unión del complejo EF-G•GTP al ribosoma, alcanzándose un nivel de detalle en esta determinación muy superior al descrito en los trabajos publicados hasta la fecha. En los ribosomas tratados, la translocación de mRNA y tRNAs sobre el ribosoma no está inhibida en sí misma, pues sí ocurre cuando es promovida por esparsomicina (Figura 5, página 112). La que está impedida es la unión del EF-G, cuya actividad GTPasa cataliza ese movimiento en condiciones normales. De nuevo se observa

la distinta interacción del ribosoma con ambos factores de elongación. En las condiciones en las que se encuentra el SRL tras la acción de la α-sarcina sólo uno de ellos, el EF-Tu, reconoce y se une al ribosoma, indicando que la conformación del SRL afecta de forma mucho más dramática a la unión de EF-G que a la del complejo ternario en el que se incluye el EF-Tu.

Desde otro punto de vista, estos experimentos también demuestran que la α-sarcina compite con los factores de elongación a la hora de unirse al ribosoma. De hecho, la porción 11-16 de la α-sarcina presenta una gran similitud de secuencia con una región del factor de elongación 2 (EF-2) (Figura 16) y probablemente esta porción 11-16 de la α-sarcina tiene un papel determinante en el reconocimiento del ribosoma. Para evaluar esta hipótesis se está construyendo un mutante en el que se remplace la región 11-16 de la ribotoxina por los residuos equivalentes de estos factores. De acuerdo con el alineamiento de secuencia que se muestra en la figura 16, se planea sustituir la Gln 10 y la Lys 11 de la α-sarcina por sendas Phe, que es el residuo que suele aparecer en esa posición en los factores de elongación. Simultáneamente, y por las mismas razones, también se sustituirá la Asn 16 por Lys.

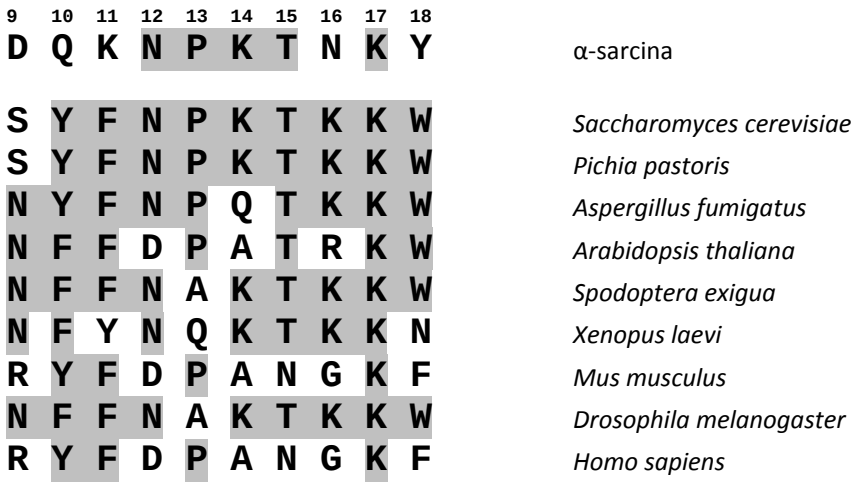

Figura 16. Comparación de las secuencias de la α-sarcina y el factor de elongación 2 (EF-2) de distintos organismos. Aparecen sombreados los residuos conservados en cuatro o más secuencias.

POSIBLES APLICACIONES CLÍNICAS DE LAS RIBOTOXINAS

Como se ha comentado repetidamente, las ribotoxinas fueron descubiertas gracias a sus propiedades antitumorales y quizás el final más deseable para todo el tiempo que se ha dedicado a su estudio sea su utilización en biomedicina. Dentro de esta perspectiva, se contemplan dos posibilidades como las más viables. La más obvia es llegar a una posible terapia antitumoral a partir de las ribotoxinas, previsiblemente utilizando α-sarcina, que es la que mejor se conoce. La otra se deriva de que otra ribotoxina, Asp f 1, constituye un alérgeno principal de *Aspergillus fumigatus*. Dado que éste es el hongo responsable de la mayor parte de las infecciones y alergias fúngicas causadas en humanos, se planteó la posibilidad de usar las ribotoxinas, o variantes de éstas, en diagnosis o terapias inmunomoduladoras para casos de hipersensibilidad a *Aspergillus*.

Para poder llegar a utilizar las ribotoxinas en terapias antitumorales es necesario aumentar su especificidad por las células transformadas y así disminuir su indeseable efecto tóxico sobre las células sanas. La principal estrategia en este sentido es la producción de inmunotoxinas, como se explicó en la introducción de esta tesis. En lo que se refiere a la porción inmunológica de estas moléculas, los últimos esfuerzos se han dirigido hacia la disminución del tamaño, pues así se simplifica el proceso de producción de la molécula por su mayor estabilidad y además le permite a la inmunotoxina una mejor penetración en tumores sólidos y una salida más fácil de los vasos sanguíneos. Así, la segunda generación de inmunotoxinas utiliza una cadena simple que contiene únicamente los dominios variables del anticuerpo, donde reside la capacidad de reconocimiento del antígeno (Brinkmann, 2000; Kreitman, 2003; Li *et al.*, 2004). En cuanto a la porción tóxica, las ribotoxinas son prometedores candidatos, precisamente por el profundo conocimiento que se tiene de ellas tras años de estudio y, de hecho, se ha dedicado otra Tesis Doctoral a desarrollar esta idea (Carreras-Sangrà, 2009). Las versiones de inmunotoxinas basadas en la α-sarcina que se han producido han ido mejorando hasta llegar a una inmunotoxina de cadena simple compuesta por los dominios variables del anticuerpo monoclonal B5 unido a la α-sarcina a través de un péptido que contiene una diana de la proteasa furina (Lacadena *et al.*, 2005). En la actualidad, se está aplicando el conocimiento adquirido en las ribotoxinas para, mediante ingeniería genética, obtener variantes de esta inmunotoxina con una estabilidad y afinidad aumentadas.

Por otra parte, ya se lleva trabajando varios años con el fin de conseguir una posible aplicación de las ribotoxinas en el tratamiento y/o prevención de hipersensibilidad a *Aspergillus*. Una de las últimas estrategias que se están investigando para el tratamiento de la alergia y el asma es la llamada inmunoterapia alérgeno-específica (SIT: allergen-specific immunotherapy). Se basa en que una exposición continuada a una molécula con capacidad inmunogénica provoca la activación de mecanismos de tolerancia que evitan el desarrollo de una respuesta alérgica. Alcanzar esta tolerancia puede requerir un tratamiento de varios días a varios meses, pero los efectos se prolongan durante años (Durham *et al.*, 1999). Este tratamiento es aún más interesante si se considera que numerosos estudios han probado que también disminuye la sensibilización a nuevos alérgenos así como el riesgo de nuevos procesos asmáticos, pudiendo emplearse también como vacuna (Des *et al.*, 1997; Purello-D'Ambrosio *et al.*, 2001). La tolerancia se induce por distintos

mecanismos. Se reduce el reclutamiento de mastocitos, basófilos y eosinófilos en la piel, la nariz, los ojos y la mucosa bronquial. Además, aumentan los niveles de anticuerpos IgA e IgG4 específicos contra el alérgeno, que evitan la unión de las IgE, que a su vez también se ven disminuidas. Por otra parte, se activan células T reguladoras, que producen citoquinas que atenúan las respuestas de tipo T_H2 (Holgate y Polosa, 2008; Larché, 2007). El factor limitante de la SIT son las reacciones anafilácticas que los alérgenos administrados pueden producir, con una incidencia del 0.1 al 5.0 % de los casos (Williams *et al.*, 2004). Para disminuir estos efectos secundarios se trabaja con alérgenos modificados químicamente (alergoides) (Lund *et al.*, 2007), con péptidos derivados de alérgenos (Larché, 2007), con alérgenos recombinantes con una capacidad de unión a IgE reducida (hipoalergenos) (Valenta y Niederberger, 2007), etc.

Obviamente, para llegar a estas terapias específicas para cada alérgeno, la diagnosis debe también avanzar en ese sentido. De hecho, la tendencia en la diagnosis de patologías relacionadas con Aspergillus es usar alérgenos purificados obtenidos de forma recombinante (Crameri *et al.*, 1998; Kurup *et al.*, 2006) en lugar de la práctica más común actualmente, el empleo de extractos de *A. fumigatus* sin estandarizar y constituidos por una variedad grandísima de compuestos (Piechura *et al.*, 1983) que llevan a diagnósticos incompletos e incluso erróneos. Así, en un diagnóstico ideal no sólo se conoce la fuente alergénica que provoca la hipersensibilidad sino exactamente las moléculas a las que cada determinado individuo es sensible. De esta forma se posibilita desarrollar tratamientos personalizados, más eficaces y seguros. La expresión heteróloga del alérgeno Asp f 1 en *Escherichia coli*, así como el método para su purificación están perfectamente establecidos. De hecho, Asp f 1 fue el primer alérgeno recombinante testado *in vivo* (Moser *et al.*, 1992), y presentó una total concordancia con las determinaciones serológicas (Moser *et al.*, 1992; Crameri *et al*., 1998; Hemmann *et al.*, 1999), por lo que podría ser empleado en este tipo de diagnosis.

La aceptación de una molécula como hipoalergénica requiere de un gran número de estudios tanto a nivel molecular como celular. La primera aproximación, a nivel molecular, con resultados prometedores, surgió al estudiar a la α-sarcina como variante natural de Asp f 1, y a los mutantes de deleción de la horquilla amino-terminal de ambas ribotoxinas (García-Ortega *et al.*, 2005a). Las características de esta región, polar, protuberante y con una estructura relativamente independiente del resto de la proteína, hacían pensar que albergaría una región inmunológica importante, pudiendo constituir un epítopo de la molécula. Efectivamente, el mutante α-sarcina Δ(7-22) dio los mejores resultados, mostrando una reducción del 50% de la reactividad a IgE a la vez que mantenía la prevalencia en el suero de los pacientes alérgicos a Asp f 1 (García-Ortega *et al.*, 2005a). Considerando que este mutante de deleción carece casi por completo de actividad citotóxica (García-Ortega *et al.*, 2002), sus posibilidades para ser usada en aplicaciones clínicas aumentaron notablemente. En esta Tesis se ha dado un paso más en el largo camino necesario para llegar a un uso clínico, la evaluación *in vivo* de la aparente hipoalergenicidad de α-sarcina Δ(7-22) (Álvarez-García *et al.*, 2009).

Para comparar la alergenicidad *in vivo* del mutante α-sarcina Δ(7-22) con la de Asp f 1 fue necesario establecer previamente un modelo múrido de sensibilización a Asp f 1. Las mejores

respuestas inmunes obtenidas en ratón se han obtenido siempre combinando una sensibilización sistémica con provocaciones repetidas de las vías respiratorias que produzcan su inflamación. Esto suele conseguirse mediante inyecciones (subcutáneas o intraperitoneales) del alérgeno adsorbido en hidróxido de aluminio, un coadyuvante bien conocido que induce respuestas Th2, seguidas de una exposición repetida de las vías respiratorias, que se consigue con un aerosol del alérgeno, o aplicándoselo de forma intranasal o intratraqueal a los ratones. Por lo general, estos protocolos inducen una respuesta inmune e inflamatoria en las vías respiratorias máxima y reproducible (Herz *et al.*, 2004). La administración a ratones BALB/c de este tratamiento con Asp f 1 recombinante purificada no provocó ningún tipo de respuesta inmunológica. Tampoco lo hizo la administración de una suspensión de *A. fumigatus* obtenida a partir de un preparado comercial del hongo. Sólo la administración de una mezcla con ambos componentes indujo una respuesta inmune en los ratones, caracterizada por altos niveles de IgE total en suero, lesiones histológicas en pulmones y fosas nasales y pérdida de peso. En cambio, como ya habían descrito otros autores (Svirshchevskaya *et al.*, 2004) no se detectaron niveles altos de IgE, IgG_1 ni IgG_{2a} específicas anti Asp f 1. Muy probablemente las moléculas de IgE total que se observan como consecuencia del protocolo de sensibilización son, en efecto, específicas de Asp f 1, pero esta especificidad no puede confirmarse por ELISA pues la concentración de IgG en suero es mucho mayor que la de IgE (Plebani *et al.*, 1986). Así, en la detección de esta última se puede producir una competencia entre ambos anticuerpos por la unión al alérgeno, lo que llevaría a subestimar los niveles de IgE específica (Adel-Patient *et al.*, 2000). Por otra parte, la sinergia observada entre Asp f 1 y el extracto de *A. fumigatus* podría explicarse por la presencia en este último de otras toxinas, o de esterasas o proteasas que eventualmente podrían actuar como coadyuvantes de Asp f 1, quizás afectando al epitelio de manera que la permeabilidad al alérgeno se vea aumentada (Kurup y Grunig, 2001; Reed y Kita, 2004; Shen *et al.*, 2007).

El modelo de sensibilización a Asp f 1 establecido se empleó para estudiar la alergenicidad del mutante α-sarcina Δ(7-22). Para ello, en la segunda fase de la sensibilización, en la que las vías respiratorias se exponen al alérgeno, se administró este mutante en vez de Asp f 1 nativa. Los daños histológicos observados fueron notablemente más leves que los producidos por Asp f 1. En los pulmones, tanto la hiperplasia de las células epiteliales como la infiltración celular perivascular y peribronquial se vio considerablemente disminuida (Figura 3, página 133). Y en las fosas nasales, el mutante provocó una disminución importante de la producción de moco (Figura 4, página 134). En cambio, los niveles de IgE de los ratones tratados con α-sarcina Δ(7-22) fueron muy similares a los de los animales tratados con una cantidad equivalente de Asp f 1 (Figura 2, página 133). Esto no debe considerarse un resultado negativo, de hecho, era de esperar, pues ni siquiera esta dosis equivalente del alérgeno nativo, Asp f 1, fue suficiente para provocar un aumento en los niveles de IgE total en suero (Figura 2, página 133). A pesar de este inconveniente, estos estudios *in vivo* han proporcionado resultados esperanzadores pues confirman la menor toxicidad del mutante, imprescindible para su potencial uso clínico, y constituyen nuevas pruebas de su menor alergenicidad. Por supuesto, se requieren estudios más profundos, que confirmen la efectividad y seguridad de esta molécula para el tratamiento de pacientes alérgicos, pero cada vez existen más evidencias de que la α-sarcina Δ(7-22) es un firme candidato a ser usado como molécula hipoalergénica en el tratamiento clínico de la hipersensibilidad a *Aspergillus*. Incluso, si fuera

necesario y ya que se dispone de la metodología, se podría continuar el estudio, tomando como base el mutante de deleción ya caracterizado, y eliminando o modificando otras zonas como el bucle 5, también interesante desde el punto de vista de su funcionalidad y alergenicidad.

En el caso de que efectivamente se admitiese el uso de alguno de estos hipoalergenos de Asp f 1, habría que plantearse el modo de administración de estas moléculas. En general, la vía oral resulta más sencilla y más segura que una ruta de administración sistémica y, además, puede inducir una respuesta inmune en las mucosas del tracto digestivo. En cambio, presenta las dificultades derivadas de las numerosas barreras que supone el tracto digestivo, lo que obliga a proteger de alguna forma el fármaco (Lavelle and O'Hagan, 2006). Con este objetivo, se han desarrollado distintos sistemas, entre los que se encuentran los biofármacos (del inglés "biodrugs"), que hacen referencia a la manipulación genética de los microorganismos apropiados para la producción *in vivo* de fármacos (Steider *et al.*, 2000), agentes antimicrobiales (Beninati *et al.*, 2000) y vacunas (Shaw *et al.*, 2000), así como para la inducción de tolerancia en enfermedades autoinmunes o alérgicas (Maassen *et al.*, 1999). El desarrollo de herramientas de ingeniería genética llevó hace tiempo al uso de microorganismos modificados genéticamente para producir fármacos a gran escala en biorreactores (Primrose, 1986). Hace pocos años ha surgido esta extensión innovadora, la producción del fármaco directamente en el aparato digestivo del receptor por la ingestión de microorganismos recombinantes vivos. Algunas ventajas de esta forma de administración en comparación con sistemas clásicos consisten en la posibilidad de administrar fármacos sensibles a las secreciones digestivas, de dirigir los fármacos a zonas específicas del tubo digestivo y de ocasionar efectos terapéuticos con dosis bajas del fármaco (Blanquet *et al.*, 2001). Con este propósito se han estudiado distintas bacterias y levaduras recombinantes, principalmente bacterias del ácido láctico (Chang y Prakash, 1998; Corthier y Renault, 1999). Este grupo de bacterias Gram positivas, que no corresponde a una clase filogenética sino a una clasificación fenotípica, pues todas producen ácido láctico al fermentar azúcares, se han empleado desde hace siglos en la producción de productos lácteos, lo que ya da idea de su inocuidad. Poseen el estatus de G.R.A.S. (del inglés "*generally regarded as safe*") y, además, algunas cepas ejercen efectos probióticos (Foligne *et al.*, 2007; Rosenfeldt *et al.*, 2003). Todas estas consideraciones situaron a *Lactococcus lactis* en una buena situación para elegirla como vehículo de ribotoxinas y posibles hipoalergenos. A pesar de que sólo un porcentaje de *L. lactis* sobrevive al paso por el duodeno (Drouault *et al.*, 1999) se ha demostrado en varios casos que estos microorganismos son efectivos en hacer llegar moléculas al colon (Steidler *et al.*, 2000) o antígenos a la mucosa del tubo digestivo induciendo respuestas inmunes locales (Robinson *et al.*, 1997; Vaassen *et al.*, 1999; Adel-Patient *et al.*, 2005; Pérez *et al.*, 2005). Por todo esto se consideró a la bacteria *L. lactis* como un vehículo ideal para la administración de Asp f 1 y/o moléculas hipoalergénicas, y se obtuvieron cepas recombinantes productoras de Asp f 1, α-sarcina, los respectivos mutantes de deleción Δ(7-22) y un mutante inactivo de la α-sarcina (H137Q) sin actividad ribonucleolítica ni citotóxica.

Por supuesto, no debe descartarse la posibilidad de utilizar también las cepas de *L. lactis* recombinantes que se han obtenido para hacer llegar la α-sarcina silvestre a tumores localizados en el tubo digestivo, más concretamente, en el colon. Según un informe de la Organización Mundial de la Salud de julio de 2008 el cáncer colorrectal causó 677000 muertes en 2007 siendo, tras el de mama y pulmón, el tercer cáncer más frecuente en el mundo, y el segundo, si sólo consideramos los que llegan a producir la muerte del paciente. La posibilidad de usar α-sarcina como agente antitumoral vuelve a ser contemplada en este contexto, pues quizás este sistema de administración solvente los problemas de citotoxicidad inespecífica de las ribotoxinas. Por un lado, *L. lactis* se dirige únicamente al tubo digestivo y allí secreta la α-sarcina, sobre todo en los últimos tramos de éste, donde el pH es más alto (Figura 2, página 145). En el estómago y las primeras porciones del intestino, la supervivencia de *L. lactis* es baja, como ya se ha comentado, pero además el pH ácido de estas regiones inhibe la secreción de α-sarcina y provocaría la inactivación de la poca que se pudiera producir (Figura 2, página 145). A diferencia de otras bacterias lácticas, *L. lactis* no coloniza el intestino y parece no tener capacidad de replicación *in vivo* en una situación fisiológica normal (Klijn *et al.*, 1995; Drouault *et al.*, 1999), características esenciales si se va a administrar intragástricamente una cepa con capacidad secretora de α-sarcina. Con vistas a explorar esta posibilidad, la inocuidad de esta administración se analizó en ratones sanos. Durante 14 días consecutivos se administró una suspensión de *L. lactis* productor de α-sarcina por vía intragástrica a ratones BALB/c. El análisis histológico reveló que las características de su tubo digestivo fueron idénticas a las de ratones control no tratados (Figura 3, página 146), sugiriendo que es posible un tratamiento continuado sin efectos deletéreos sobre los ratones. Resta, por tanto, evaluar directamente este sistema en el tratamiento de tumores gastrointestinales y para ello lo ideal es usar un modelo animal de esta patología. El cáncer colorrectal es tan frecuente en humanos que el número de estos modelos disponibles, sobre todo en ratón, es muy alto. Entre todos ellos, resulta muy adecuado un modelo genético de ratones de la cepa C56BL/6J en el que la mutación APC^{Min}, autosómica dominante, predispone a los ratones a desarrollar adenomas en el tracto intestinal. Los ratones heterocigotos para esta mutación desarrollan a lo largo de todo el intestino más de 50 adenomas que evolucionan hasta tumores benignos, sin capacidad de producir metástasis. Este modelo resulta, pues, muy conveniente para probar la capacidad de la α-sarcina secretada por *L. lactis* en la inhibición del crecimiento de tumores y su utilización con este propósito se encuentra ya en desarrollo durante el proceso de redacción de esta Memoria.

CONSIDERACIONES FINALES

La actividad antitumoral de las ribotoxinas provocó su descubrimiento en los años 50, suscitando importantes expectativas en cuanto a su potencial uso clínico, pero su estudio se abandonó completamente al desvelarse su citotoxicidad inespecífica. Poco tiempo después, y tal vez desde un punto de vista más académico, volvieron a resultar interesantes por su exquisita actividad ribonucleasa, que inactiva al ribosoma mediante la hidrólisis de un único enlace fosfodiéster entre los más de 7000 presentes, y por su capacidad adicional de atravesar membranas celulares. Paradójicamente, tras años de estudio de sus características estructurales, de sus propiedades enzimáticas y de su interacción con membranas lipídicas se ha llegado a un conocimiento tan exhaustivo de estas proteínas que su uso en aplicaciones biomédicas ya no puede descartarse. En esta Tesis aparece una pequeña muestra de ello. Se ha profundizado en las relaciones estructura-función de estas proteínas, estudiando su centro activo y el de las RNasas microbianas extracelulares en general, donde las ribotoxinas se incluyen. También se ha analizado si el carácter básico de la horquilla β amino-terminal es determinante en su función de reconocimiento del ribosoma y de interacción con fosfolípidos. Y se ha estudiado el efecto de las ribotoxinas sobre la unión y actividad de los factores de elongación en el ribosoma. Por último, se ha utilizado todo el conocimiento acumulado para plantear dos aproximaciones para el uso de las ribotoxinas en aplicaciones clínicas. Por un lado, se ha tratado de evaluar in vivo el posible carácter hipoalergénico de determinados mutantes de la ribotoxina Asp f 1, estableciendo previamente un modelo de hipersensibilidad a esta proteína en ratón. Por otra parte, se ha propuesto una nueva vía de administración de ribotoxinas o variantes de ellas al tubo digestivo, empleando la producción heteróloga de una bacteria láctica, *Lactococcus lactis*.

En resumen, el trabajo con las ribotoxinas constituye un buen ejemplo de cómo en algunos casos los estudios y descubrimientos de la investigación básica pudieran llegar a dar lugar a aplicaciones clínicas insospechadas inicialmente.

Conclusiones

Las conclusiones generales que pueden extraerse de los resultados presentados en esta Tesis Doctoral son:

- La Tyr 48 de la α-sarcina es un residuo del centro activo esencial en su especificidad, resultando clave en el reconocimiento de sustratos de RNA polimérico.

- La His 101 de la RNasa U2 es un residuo fundamental para el correcto plegamiento de la proteína, al menos cuando se expresa en la levadura *Pichia pastoris*.

- La eliminación del puente disulfuro que se establece entre los residuos 1 y 54 de la RNasa U2 permite su correcto procesamiento postraduccional y evita la producción de formas incorrectamente plegadas en *P. pastoris*.

- Parte de la funcionalidad de la horquilla β amino-terminal en la citotoxicidad de la α-sarcina reside en su carácter básico. Los residuos de Lys y Arg de esta horquilla establecen interacciones electrostáticas claves tanto para el reconocimiento del sustrato como para la interacción con bicapas de fosfolípidos ácidos.

- La ruptura del SRL por parte de la α-sarcina dificulta la unión del complejo ternario al ribosoma, reduce la unión del factor de elongación G y por ello inhibe muy significativamente su actividad para hidrolizar GTP, impidiendo la translocación e inhibiendo así la biosíntesis de proteínas.

- Se ha optimizado la inducción de un modelo de alergia a Asp f 1 en ratones. Este modelo es, asimismo, mucho más fácilmente reproducible que los descritos hasta la fecha.

- Utilizando el mencionado modelo, se ha verificado *in vivo* la menor alergenicidad del mutante de deleción α-sarcina Δ(7-22) en relación con el alérgeno Asp f 1.

- Se han obtenido cepas de *Lactococcus lactis* capaces de producir y secretar el alérgeno Asp f 1 y tres variantes con afinidad reducida por IgE.

- Se ha demostrado que la administración intragástrica durante 14 días de la cepa de *L. lactis* modificada para producir α-sarcina silvestre resulta inocua para ratones sanos.

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